INDUCED VARIATION IN *PUCCINIA STRIIFORMIS* WESTEND., AND STUDIES OF RELATED CYTOLOGICAL AND DEVELOPMENTAL PROCESSES

A dissertation submitted to the

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by

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PREFACE

This dissertation is a result of work done at the Plant Breeding Institute, Trumpington, Cambridge, between October 1970 and October 1973. The data and conclusions are mine except where duely acknowledged. No part of this work has been previously published or submitted to any other University for a degree.

I wish to thank Dr. R. Johnson for his supervision throughout this work, Dr. P. Wooding for his assistance with the electron micrographs, Mr. B. Allen for his assistance with the photography, and many others for advice and encouragement during this work.

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SUMMARY

Yellow rust of wheat, Puccinia striiformis Westend., is one of the major cereal pathogens of Northwest Europe and cold temperate to temperate climates, causing considerable loss in yield and reduced grain quality.

One of the important features of this pathogen is its ability to overcome the resistance of many commercial varieties of wheat, by the development of new physiologic races with different virulence characters. Since no sexual stage is known in this fungus, it is assumed that changes in virulence arise by mutation or asexual recombination.

These studies were carried out to investigate the occurrence of variation in controlled experiments, and some possible modes of origin of this variation, including the parasexual cycle and whole nuclear reassortment.

Mixed race infections, using races 41 E 136 and 104 E 137, were established in glasshouse experiments, and single spore isolates were obtained from these infections. Three isolates were obtained which exhibited the combined virulence of the two races, giving a previously unrecorded race, 105 E 137. One isolate of this new race had a larger spore size than did the 'parental' races, and this is discussed in relation to the possible operation of the parasexual cycle.

Considerable variation was detected in isolate generation time, and this was found to be correlated with reaction type, as susceptible reactions achieved sporulation in a shorter time than did intermediate-type reactions. Some variation between isolates on susceptible varieties was also detected, which suggested that generation time could be used as an indication of host-pathogen compatibility.

Histological studies were undertaken in order to investigate factors which could have influenced the occurrence of the observed variation. Three types of host pathogen interaction were studied, associated with resistant (00), intermediate (0), and susceptible (4) reactions. These resulted in differing internal environments which influenced the size of the fungal colony within the leaf, and this is discussed in relation to the likelihood of new race formation by contact between colonies of two different races.

Observations of hyphal fusions, within the leaf, and between germ tubes on leaf and agar surfaces were obtained. Large numbers of nuclei were observed in germ tube fusion bodies, and this suggested that nuclei were able to pass from their germ tube of origin, and possibly take part in genetic reassortment.

Measurements of nuclei and nucleoli revealed sizes of 4.0 x 3.1 μm and 1.5 x 1.3 μm respectively, which are larger than previously reported. The chromosome number was established as n = 6, which would suggest that P. striiformis is heterothallic.

A method is described by which spore nuclei from two races can be distinguished microscopically, but conclusive evidence of nuclear exchange was not obtained.

Some progress was made in attempts to grow P. striiformis in axenic culture, in order to provide fungal mycelium free of host tissue for cytological studies.

CONTENTS

GENERAL INTRODUCTION

Yellow rust of wheat, Puccinia striiformis Westend., is one of the major cereal pathogens of North West Europe and cold temperate to temperate climates, though its range is now extending to warmer and more arid regions, e.g. Yugoslavia (Spehar, 1966), and Iran (Bamadadian, 1972). Manners (1950) has shown that it is the distribution of susceptible wheat, rather than climatic and topographical factors, which controls the distribution of the pathogen. Macer (1972) pointed out that changes in the physiologic race spectra take place following the introduction of resistant varieties into cultivation, either by multiplication of virulence factors already present at low frequency in the fungal population, or by their formation de novo.

With an increasing acreage of rust resistant wheat varieties, the fungus is being forced to produce more new races capable of attacking these varieties, in order to survive. It is evident from the known races, of which nine new races and two new race variants have been identified in Britain over the past 20 years, that the fungus is capable of changes in virulence.

Black rust of wheat, Puccinia graminis tritici, illustrates the complete life cycle associated with the genus Puccinia. It has an asexual cycle occurring on wheat and a sexual cycle on a secondary host, in this case Berberis vulgaris. This means that variation can arise sexually or asexually. However the secondary host of P. striiformis is not known and consequently the sexual cycle, if present, cannot be investigated, which means that the only variation which can be studied is asexual.

These studies are to investigate the occurrence of variation in P. striiformis in controlled experiments, and some of the possible modes of origin, including the parasexual cycle and heterokaryosis.

I. PHYSIOLOGIC SPECIALISATION OF PUCCINIA STRIIFORMIS

I1. Literature Review.

1a. Nomenclature

Puccinia striiformis Westend., was first described by Eriksson and Henning in 1896, but the occurrence of yellow rust in Britain had been recorded by J.S. Henslow in 1841. He described the orange-yellow infection of the stem, leaf and chaff, followed by a deep brown stage. There is little doubt that this was P. striiformis, and not, as he reported, a stage of Uredo linearis (= P. graminis) (Wilson and Henderson, 1966). He later reported both yellow and brown sori inside the glumes and included drawings of both uredo- and teleuto-spore types.

The uredial stage was originally described by Schmidt in 1818, and called Uredo glumarum, but in 1854 Westendorp observed the telial stage, and placed it in the genus Puccinia. The name Puccinia glumarum was used from 1896 to 1956, when Cummings and Stevenson introduced the name Puccinia striiformis in their check list of North American rust fungi. This is the generally accepted name at the time of writing.

1b. Effect on yield

Rust attack affects yield primarily by reducing the carbohydrate supply to the developing parts of the plant, especially to the ear (Doodson et al., 1965). Batts (1957) estimated that severe P. striiformis infections could result in reductions in yield of up to 50% in very susceptible winter wheat varieties. Doling and Doodson (1968) recorded losses of up to 20% from field experiments, which was comparable to Batts and Elliott's (1952) observation of 22% loss on the variety Wilma. Doodson et al. (1964) recorded a 59% loss in yield in glasshouse experiments when foliage and ears were severely infected. Further experiments indicated that a severe ear infection

causes an additional loss in yield equivalent to half that of foliar infection. Doling and Doodson (1968), using the hypothesis above estimated that the maximum loss of yield in the field would be in the order of 45% which corresponds with the estimated losses in some commercial crops in 1966.

In addition to the loss in yield, the grain quality is greatly reduced, resulting in shrivelled grain which reduces still further the total yield of good quality grain.

1c. Chemical control

The chemical control of rusts is promising (Rowell, 1964) but would only be economical if an efficient forecasting system was in operation, as at present fungicides are not efficient for a sufficiently prolonged period without repeated applications. Zadoks and Rijsdijk (1972) described a method by which a wheat yellow rust epidemic can be described and possibly in the future be predicted. The system, called EPISIM, uses available data on rain, temperature, dew and crop development. It is thought that the system is adaptable to other rusts when the necessary quantitative relationships are available.

1d. Distribution and epidemiology

Puccinia striiformis has a low temperature optimum of 10°-15°C, depending on the stage of development. This accounts largely for its geographical distribution in the colder climates of North West Europe, though recently yellow rust has been active in several Mediterranean countries. These observations do not seem to agree with environmental requirements. No definite explanation can be put forward, but there is some evidence that some biotypes are better adapted to higher temperatures

than others, and that the high temperature induced type of resistance present in many cultivars in temperate climates may be missing in some of the circum-Mediterranean cultivars, (Zadoks, 1965).

On the continent of Europe black rust, P. graminis, overwinters on barberry (Berberis vulgaris), but Ogilvie and Thorpe (1961) have established that P. graminis does not overwinter on barberry in Britain, and that epidemics, when they do occur, are caused by spores blown in from the continent and Mediterranean regions. In P. striiformis, however, there is no secondary host. The uredospores are frost hardy (Mehta, 1923) and there is evidence that the pathogen can overwinter in the uredial state on winter wheat, and that the rust can survive for periods up to 5 months, especially under snow cover, (Zadoks, 1961). These isolated infection foci may be the source of inoculum for annual rust attacks, given favourable conditions, although race distribution, e.g. the spread of race 60 to Holland (WMO '69) in 1966/67 indicates that long distance spread can occur.

P. striiformis is a windborne pathogen, and international dispersal of inoculum can take place, (Stubbs and Fuchs, 1965). However, Maddison and Manners (1972), have shown, using criteria of spore germination and the ability to infect, that P. striiformis is $1\frac{1}{2}$ times as sensitive as P. recondita and $2\frac{1}{2}$ times as sensitive as P. graminis to the effect of U.V. radiation. They suggest that P. striiformis spores would only remain viable for a period of 1 day if exposed to U.V. radiation during the summer months, thus limiting the distance of viable spore dispersal to the distance travelled within one day. This would, however, allow a step-like spread and gradual build up of infection as outlined by Zadoks (1965) in his description of the development of an epidemic on wheat and barley which affected much of N.W. Europe in 1961.

1e. Physiologic specialisation

Physiologic specialisation was first demonstrated in P. striiformis in 1930, (Allison and Isenbeck, 1930). In 1932 Gassner and Straib established a set of eleven differential varieties on which the different races gave consistently different reaction types. This meant that races could be identified by comparing their reaction types on these varieties.

Manners (1950) identified 8 races of P. striiformis which attacked wheat in Britain, using the differential varieties of Gassner and Straib. He suggested that race 6 was complex, and required additional varieties to differentiate between the isolates classified as race 6.

In 1952, there was an extensive outbreak of yellow rust in Holland, and the following year, Nord Desprez, previously resistant, was attacked in Britain. Batts (1957) attributed these outbreaks to the new race 2B, which was comparable with the German race 2x, (Fuchs, 1960) and similar to race 2 with added virulence on Cappelle-Desprez and Nord Desprez. Batts (1957) observed that race 2 had not been identified since 1953, and suggested that race 2B had completely replaced it.

Batts (1957) also identified race 8B on the variety Heines VII. This race could only be distinguished from race 8 on Heines VII and some additional differential varieties. It had been detected in glasshouse tests during 1954 (Batts, 1957) in isolates of race 8, prior to its widespread occurrence in 1955 and 1956 on Heines VII.

The variety Rothwell Perdix was severely attacked by rust in 1966. Race 60 was isolated and identified, and it was suggested (Macer and Doling, 1966) that it could have arisen from any of three races, 8B, 27/53 and 54. Race 60 is similar to 27/53 with added virulence on Heines Kolben and Chinese 166; and similar to race 54 with increased

virulence on Chinese 166 and Heines VII. It was postulated that race 8B was the most likely parental source, based on the wide distribution of this race in Britain at the time.

In 1969 however, races 3/55D and 58C were isolated and identified from field plots (Johnson et al., 1969; Chamberlain et al., 1970 and 1971). It was found that race 58C could attack Rothwell Perdix but not Heines Kolben, which suggested that virulence on Heines Kolben was unnecessary for virulence on Rothwell Perdix. It was therefore suggested that race 60 could have originated from the less common race 54.

Race 58C was similar to race 58 (Macer, 1967) in having the combined virulence on Vilmorin 23 and Chinese 166. It differed from race 58 in that it was able to attack the varieties Heines VII, Minister, Professor Marchal, Rothwell Perdix and Cama. Race 58C is also capable of attacking Cappelle Desprez. This means that it has overcome the resistance genes Yr 1 (Chinese 166), Yr 2 (Heines VII), Yr 3c (Minister) and Yr 3a and 4a (Cappelle Desprez) (Lupton and Macer, 1962), which are common in many commercial varieties. It is unable to attack the varieties Hybrid 46 (Yr 3b and 4b), Triticum spelta (Yr 5), Heines Peko (Yr 6) and Thatcher (Yr 7), or varieties containing these resistance genes (Macer, 1966b) separately or in combination. It has been suggested (Chamberlain et al., 1970) that a single step mutation on Chinese 166 of race 3/55 Cleo (Stubbs et al., 1966) present in Britain at the time could have given rise to the new virulence combination.

Race 3/55D is similar to races 3/55 Opal (Stubbs, 1964) and 3/55 Cleo, with the added virulence on Maris Beacon. An identical isolate had been obtained from plots of Maris Beacon at the Plant Breeding Institute in 1967, but it was at very low levels and only collected from this one location.

In 1966 a race containing the virulence combination capable of infecting the varieties Vilmorin 23 and Heines Kolben was recorded (Chamberlain et al., 1971). This virulence combination had not previously been recorded in Britain, but was found to be similar to race 1 (Fuchs, 1965). After tests with supplementary varieties Hybrid 46 and Maris Ranger, to which it was virulent, and Heines VII, Heines Peko, and Cama to which it was avirulent, it was designated race 1B.

Two variants of races 3/55D and 58C were isolated from Joss Cambier in 1971. The two variants were not distinguishable from the previous isolates on the standard differential varieties, but the 3/55D variant appears to be better adapted to Joss Cambier than to Hybrid 46 (Johnson and Taylor, 1972).

1f. Race Nomenclature

A new race nomenclature was proposed in 1972 by Johnson et al. This was felt necessary (Manners, 1969) as some of the varieties in Gassner and Straib's (1932) set of differential varieties were later shown to be labile in their reaction, depending on environmental conditions, and also isolates obtained from commercial varieties were indistinguishable on the standard differentials. This led to the use of letters (Batts, 1957; Chamberlain et al., 1970 and 1971) or of commercial varieties which were susceptible to the races being used to distinguish between these isolates, (Ubels et al., 1965; Zadoks, 1961). The new race nomenclature (Johnson et al., 1972) is based on decenary values attributed to two sets of differential varieties: one set consists of 9 varieties selected as a primary or world set, which have been chosen to distinguish the broad spectrum of known variation in P. striiformis; and the other, the secondary or European set of 8 varieties has been selected from currently used European varieties. The system is open-ended in that new varieties

can be added to each set of differentials should they be required, and the second set may be separately established for a given area using locally grown varieties. Hence American workers may use a different secondary set to European workers, etc. The race numbers are obtained by adding the values attributed to each susceptible variety in each set of differentials. The first number stated is the world number, and the second one, following a suitable prefix, in the case of European isolates E, gives the European number. Thus race 41 E 136 attacks Strubes Dickkopf (32), Vilmorin (8), and Chinese 166 (1) in the world set and Heines VII (128) and Nord Desprez (8) in the European set.

Several new races have been provisionally identified* in 1970-1972, using the new nomenclature, of which 45 E 140 and 108 E 141 are two. The importance of these races will be discussed in connection with the results obtained from this work.

1g. Breeding for rust resistance

Inheritance of yellow rust resistance at seedling and adult plant stages have been of great importance to the plant breeders (Lupton and Macer, 1962; Macer, 1966a) and its exploitation by the breeders has been summarised by Macer (1972) and Manners (1969). Recently, however, there have been devastating attacks by new races on varieties which, when released, were resistant to the known races. Two examples are the attack of race 8B on Heines VII (Zadoks, 1961) and race 60 on Rothwell Perdix (Macer and Doling, 1966). Zadoks (1965) observed that the greater the success of a resistant variety, the more severe the epidemic when the resistance breaks down, and that breeding for resistance against one race has led to epidemics of another.

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* Identified by the N.I.A.B. Physiologic race survey.

With this in mind, the emphasis has changed from breeding for major gene, or vertical resistance (Van de Plank, 1963) which gives a good level of resistance to certain races, to minor gene or horizontal resistance, in which there is a uniform level of resistance to the known races. It is hoped (Lupton and Johnson, 1970) that the resistance is non race specific and should be effective to the same level against any new races formed.

1h. Origins of variation

As Puccinia striiformis lacks a known secondary host, only methods by which variation can arise asexually have been considered.

A number of possible methods by which new races can arise asexually have been proposed in the rusts including: nuclear reassortment in P. graminis tritici (Nelson et al., 1955; Watson, 1957b), M. lini (Flor, 1960 and 1964) and in P. striiformis, (Little and Manners, 1969a; Konolova and Schekotkova, 1970); the parasexual cycle (Pontecorvo, 1956) in P. graminis tritici, (Sharma and Prasada, 1969; Watson and Luig, 1958 and 1962); mutations (Watson, 1957a); and extrachromosomal inheritance in P. graminis avenae (Green and Kirmani, 1969), and P. graminis tritici (Watson, 1957b).

The production of new physiologic races as a result of reassortment of whole nuclei was first demonstrated by Nelson et al. (1955) in P. graminis. They inoculated the varieties Khapli (resistant) and Little Club (susceptible) to the races 38 and 36 with a mixture of the two races. A biotype was obtained which could attack Khapli. This was found to be 3-4 nucleate, and occurred at a frequency of about 10% in early generations, the frequency decreased with time, and gave rise to two stable avirulent binucleate biotypes. It was suggested that the virulence on Khapli was conferred by the extra nucleus.

Little and Manners (1969a) inoculated the wheat variety Strubes Dickkopf, susceptible to both races 2B and 8B of P. striiformis with a mixture of the two races, and from the 30 single spore isolates obtained, they identified two new isolates, SSC 4 and SSC 21. SSC 4 resembled 2B but gave a resistant reaction on Vilmorin 23 and Rouge Prolific Barbu instead of the expected susceptible reactions. Virulence was shown on Cappelle-Desprez, while not on Vilmorin 23, whereas previously these two varieties had reacted similarly to all known races. Culture SSC 21 also resembled race 2B but incorporated the virulence on Heines VII, associated with race 8B, thus combining the virulence on Heines VII and Cappelle Desprez.

Little and Manners point out that if P. striiformis is heterothallic like other cereal rusts (Buller, 1950), and nuclear reassortment is active, two new races rather than four from a homothallic fungus in which all four nuclei were different would be expected from the mixed inoculum. However, if two or more nuclei were similar or identical, fewer races would be expected. As two races were obtained, they assumed the fungus to be heterothallic. They did not preclude the possibility of the parasexual cycle or mutations, but as races are generally stable (Little and Manners, 1969a) in a single race culture, they favoured heterokaryosis. The new isolates showed altered virulence on the differential hosts tested. Little and Manners (1969a) suggested that the virulence on Cappelle was dominant, as both recombinants were virulent on Cappelle, despite race 8B being avirulent on Cappelle. They have discussed virulence on Vilmorin 23 and Rouge Prolific Barbu and Heines VII in both the dominant and recessive condition.

The Russians, Konovalova and Shektokova (1970), inoculated the wheat varieties Cappelle-Desprez and Heines VII with various combinations of the

races 7M, 14 and 25, to which they are resistant. A virulent isolate was obtained when Cappelle was inoculated with 7M and 14. The isolate was considered to be a heterokaryon. When compared with the other races on the differential varieties, the heterokaryon was found to be avirulent on all the varieties except Cappelle, on which it was established. Marked necrosis occurred on the varieties Michigan Amber, Strubes Dickkopf, Webster, Chinese 166 and Rouge Prolific Barbu. After three generations the heterokaryon reverted to its original form.

From the Russian work it would seem that virulence on Michigan Amber and Triticum dicoccum tritici was dominant, but in a heterozygous condition, and from this, it would seem that the avirulent heterokaryon was a homozygous recessive. Virulence on the other varieties is probably recessive. In the heterokaryon virulence in Cappelle would have been present in the homozygous recessive condition: the parental races were therefore probably in the heterozygous condition, in contrast to the observations of Little and Manners (1969a).

The Russian isolate is comparable to the 'forced heterokaryon' of Caten and Jinks (1966), having arisen under a heavy selection pressure and being unable to exist in less exacting conditions. Caten and Jinks pointed out, however, that these heterokaryons are unlikely to arise in nature: the two homokaryons must meet and anastomose in favourable conditions, and then be subjected to the conditions allowing only the heterokaryon to grow. It would therefore be advantageous to obtain a better understanding of the stage at which resistance is operational.

The isolates obtained by Little and Manners (1969a), could be compared with neutral and naturally occurring heterokaryons (Caten and Jinks, 1966), in which no selection pressure is operational. As Little and Manners observed cytoplasmic streaming, but only assumed nuclear exchange, it is possible that the isolates are heteroplasmons (Jinks, 1959b) and not

The production of more than two new races from two parental races can be explained by the parasexual cycle (Pontecorvo, 1956, 1959), which accomplishes genetic recombination and thus increases the available genetic variation.

The parasexual cycle involves the fusion of unlike nuclei in a heterokaryon, followed by mitotic crossing over and haploidisation. The reported infrequency with which the three steps in the cycle occur, 1 in 10^6 for vegetative nuclear fusion, 1 in 500 nuclear divisions for mitotic crossing over, and 1 in 1,000 nuclear divisions for haploidisation, in Aspergillus nidulans means that the cycle can be of little importance in an organism exhibiting a normal sexual cycle. It may however, be important in imperfect fungi and heterothallic fungi in which populations of only one mating type are common (Fincham and Day, 1971). A. niger exhibits all stages of the parasexual cycle, and it seems likely that the frequencies of vegetative fusion, and of diploid vegetative segregation may be greater than in A. nidulans, (Pontecorvo et al., 1953). It is probably important in the origin of new races in plant pathogenic imperfect fungi, and has been reported in Fusarium oxysporum (Buxton, 1956) and other fungi (Buxton, 1960). Tinline and MacNeill (1969) gave a comprehensive summary of work on parasexuality in plant pathogenic fungi and they pointed out that although parasexuality seems to be a fairly common occurrence in the laboratory, evidence for parasexuality in natural populations is scarce.

Sharma and Prasada (1969) obtained 6 and 8 isolates from two race mixtures of P. graminis. These numbers of different isolates are not obtainable if heterokaryosis alone is operational, whereas the parasexual cycle offers a likely explanation. Watson and Luig (1962) obtained 10 colour variants of P. graminis from mixed race inoculations on Little Club.

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They explained that nuclear exchange and mutations were unlikely to account for the variation observed, and again the parasexual cycle seems likely.

Bridgmon (1959) pointed out that the diploid phase is necessary for the complete parasexual cycle, and this has not been observed cytologically in rust fungi, (Bridgmon, 1959; Little and Manners, 1969b) except in axenic culture of P. graminis (Williams and Hartley, 1971). Ellingboe (1961) however, claimed that such a phase must exist, even if it is only of short duration, as the genetic recombination has been shown to occur.

The parasexual cycle, if operational and of frequent occurrence, should be evident in single race cultures, as some rusts are known to be heterozygous for several virulence genes, (Ellingboe, 1961) and each cell contains two compatible nuclei. Minor variations may be present in single race cultures, but there has been little evidence of major variation arising. It is possible that any such variation could be, and probably is, selected out by workers who might consider variation in a single race culture as contamination from another race, when several races are being handled at any given time.

There has been no evidence favouring the parasexual cycle as the likely source of variation in P. striiformis, but the possibility has not been totally precluded (Little and Manners, 1969a).

The role of extrachromosomal inheritance cannot be overlooked. Green and Kirmani (1969), having eliminated mutation, heterokaryosis and mitotic segregation, postulated that extrachromosomal inheritance was the most likely explanation when they obtained a culture from an orange pustule of P. graminis sp. avenae, but were unable to obtain a pure line from it.

Although the red-brown isolates established were stable, the pustules obtained from single orange uredospores segregated to give orange and red-brown pustules.

Mutations for pathogenicity have been demonstrated in P. graminis by Watson (1957a). He obtained spontaneous mutations in glasshouse studies and the four isolates differed in both colour and pathogenicity. The two orange isolates were more virulent on Lee than were the original races, though they were not distinguishable on Bowie. It is difficult to say whether they were single gene mutants, or changes involving two or more genes.

There has been no experimental evidence supporting mutations in P. striiformis, although this was proposed as a possible origin of race 58C (Chamberlain et al., 1970). Single race cultures are generally considered to be stable and can be maintained for many years in glasshouse conditions. Such spore cultures are however protected from the damaging effects of ultra-violet rays and therefore may yield exceptionally low mutation rates. Although mutations do occur, it would seem that the rate was too low to account for the frequency with which new races arise. Two colour mutants of P. striiformis have recently been discovered (Brown and Sharp, 1970) and one at the Plant Breeding Institute (P.B.I.). In both cases no change in the virulence was detected.

These studies reported here were intended to detect variation in pathogenicity and aggressiveness of isolates of the pathogen arising from mixed race inoculations, and also to try and establish a mechanism by which such variation arises.

VARIATION IN PUCCINIA STRIIFORMIS

I2. Experimental Methods and Materials.

i) Isolation systems

ia) Glasshouse (Plate I.1a). The experiments were carried out in a glasshouse ventilated with air filtered through Ozonair S-mat filters which removed particles down to 4 microns in diameter with 99.9% efficiency. The glasshouse was divided into 8 compartments opening off a central corridor into which the filtered air was blown by fans which could operate at four speeds providing a maximum of 60 air changes per hour. On leaving the compartments the air passed through Ozonair paper pleat filters to remove any spores which had entered the air stream; this was important to prevent the release of any new races of P. striiformis produced during the experiments.

Supplementary lighting was provided by banks of 11 white 125 watt 8 ft. fluorescent tubes, but to prevent excess heating by the lights they were switched off thermostatically when the ambient temperature reached 20°C. Experiments were discontinued from December to March because the supplementary lighting was insufficient to provide suitable conditions for development of satisfactory infections.

In the summer, temperatures occasionally exceeded 30°C in the glasshouse, but work was continued and allowance was made in interpreting the infection types on some varieties which became more susceptible at high temperatures.

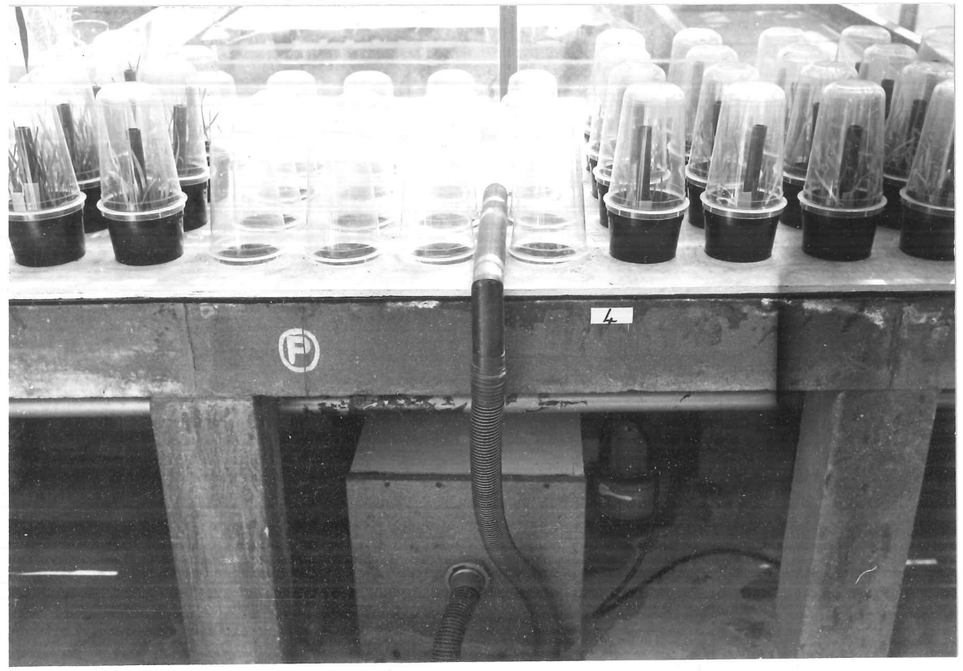
ib) Isolation bench (Plate I.1b). One glasshouse bench was modified according to a design by Jenkyn et al. (1973) to provide 80 isolation chambers each consisting of a 4.5 in (12 cm) Stewarts Plastic plant pot with a central ventilation tube (1.7 cm internal diameter) and covered

Plate I.1a. Filtered air glasshouse.

Plate I.1b. Isolation bench.

a

b



with a 14 cm Stewarts Plastic Crystal dome (Plate I.2a). A Woods 0.7 amp, 11 cm centrifugal fan was used to supply air to the bench after filtration through a Vokes H66 Absolute Filter. Initially, wicks were suspended from the base of each pot to conduct water from the bench into each pot. This provided high humidity in the isolation chambers and encouraged the growth of a hyperparasite Cladosporium uredinicola Speg. (D. Allen Pers. Comm.) on the sporulating rust pustules. The benches were drained and the wicks removed thus reducing the humidity and reducing the hyperparasite. The isolation chambers were removed from the bench to a Bassaire lamina flow filter bench (Plate I.2b) before opening for watering, twice a week during the winter and more often during the summer. The isolation system enabled up to 60 isolates to be multiplied simultaneously without cross contamination.

The effectiveness of this isolation system was illustrated using the white mutant isolate. Although up to 30 isolates were being handled in the compartment at any one time, and 10 or more isolates were inoculated on one day, there was no evidence of white or yellow isolates becoming cross contaminated.

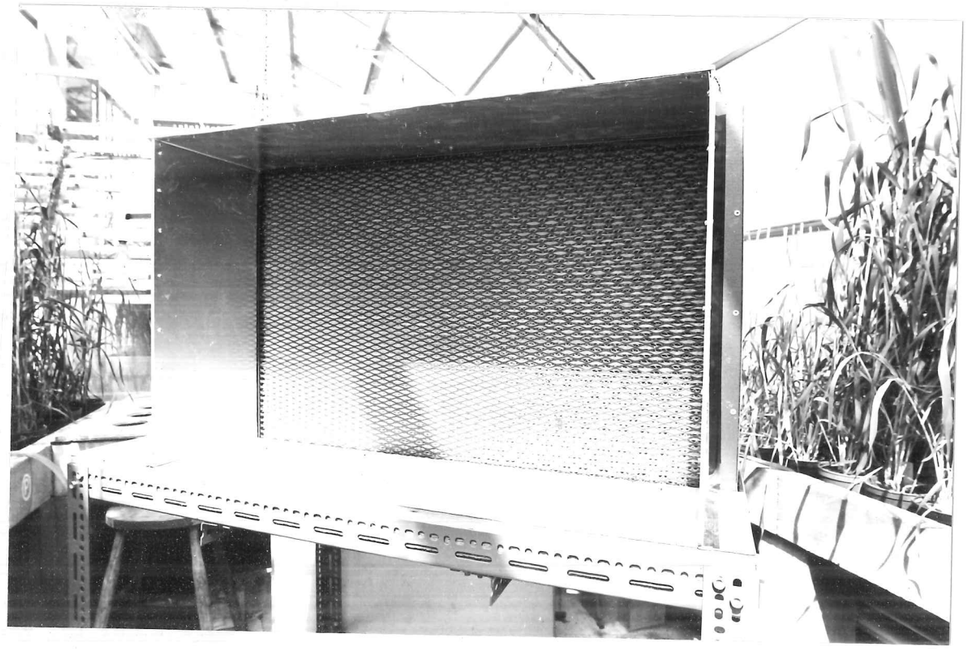
Before the isolation bench was constructed, 14 isolation chambers were used in one of the glasshouse compartments. The isolation chambers were made of a wooden frame covered with cellophane and were placed over 20 pots of infected seedlings. This system was probably effective when the covers were in place, but when the chambers were removed in order to collect the spores, the lack of a filtered air system meant that cross contamination was unavoidable.

Plate I.2a. Isolation bench pots.

Plate I.2b. Bassaire Lamina Flow filtered air bench.

a

b



ii) Races of P. striiformis used

Two races of Puccinia striiformis with contrasting virulence characteristics were chosen for this work. According to the nomenclature of Johnson et al. (1972) these were named race 104 E 137 (previous name 3/55D) and 41 E 136 (58C). Both races are currently common in Britain.

iia) Inoculations to check the purity of the rust stocks. Seedlings of Maris Beacon were inoculated with race 104 E 137 and seedlings of Maris Envoy were inoculated with race 41 E 136. Single spore isolates were established from both infections and these, along with the remaining spores from the two inoculations, were tested separately on the differential varieties. From the reaction types obtained, both spore cultures were found to be pure race cultures.

Seedlings of Jubilegem were inoculated with spores of the white mutant isolate found in an infection of race 104 E 137. The spores were collected and 7 single spore isolates were obtained. These isolates were tested, together with the remaining white spores and a yellow culture of race 104 E 137. All seven isolates and the remaining spores gave the same reactions as the yellow culture of 104 E 137, and it was established that these were pure cultures of race 104 E 137.

iii) Multiplication, collection and storage of races and isolates

Seeds of the varieties to be inoculated were sown in pots of sterile compost, and kept in a spore-free glasshouse compartment for a period of 9-14 days until the first leaf was fully expanded, when they were ready for inoculation.

iiia) Inoculation of isolation bench pots. Each 12 cm isolation bench pot was sealed and transferred to the compartment containing the isolation bench. The bags were transferred individually to the Bassaire filter bench and were unsealed. Spores were brushed onto the leaf surface, using a clean, dry paint brush. Water was added to the polythene bag, and the plants were then sprayed with a fine mist of distilled water using a Shandon Laboratory Spray Gun. The bags were re-sealed and hosed down to prevent any spores being transferred to the central corridor of the glasshouse, and were transferred to a refrigerator at 7°C overnight.

The following day, the pots were returned to the isolation bench, and a plastic dome was placed over each pot (Plate I.2a). Sufficient spores for testing and maintaining the stocks could be obtained from multiplications on one or two isolation bench pots. The parental races 104 E 137 and 41 E 136 were multiplied frequently to maintain sufficient spores for controls

iiib) Spore collection. After a period of 10-14 days sporulation occurred. Spores were collected from the isolation bench pots using a cyclone spore collector (Tervett and Cassell, 1951), while the pots were in the Bassaire filter cabinet. The cyclones were washed through with industrial spirit and left to dry in the cabinet between each collection of each isolate to prevent cross contamination.

Tests of the isolates on differential varieties were carried out in a separate compartment and spores were not collected from the testing compartment as cross contamination between sporulating isolates would have been inevitable.

iiic) Spore storage. The collected spores were stored for a few days in glass tubes over 40% sulphuric acid in a desiccator, which was kept in a refrigerator at 3°C. Any spores which had not been used during this period were vacuum dried using an Edwards Speedivac Vacuum drier (Hughes and Macer, 1964) for 2 hours. The sealed ampoules were stored at about 3°C until required.

iiid) Inoculation of test varieties. Up to 40 5 cm pots of seedlings were placed on a tray inside a metal liner, a deep, tight fitting jacket was then placed over the liner to seal the unit (Cook, 1972). The plants were transferred to the test compartment for inoculation.

Each inoculation unit was in turn opened and the plants thoroughly watered. A mixture of purified talc and spores was then dusted onto the plants. The plants were sprayed with a fine mist of distilled water, and the outer jacket was replaced. The tray was then flooded with water, to seal the inoculation unit and ensure that free water was present during the incubation period. The inoculated plants were placed in a refrigerator at 7°C overnight, as above.

The following day the pots were arranged on the glasshouse bench. They were cut back to the first leaf at weekly intervals, and scored on a 00 to 4 scale (Table I.1), after 14-16 days when the reactions were fully developed.

Each reaction type, where necessary, has been qualified with a + or -, to indicate the gradation in reaction types (Plate I.3). Reactions 0 to 2- were taken as resistant, and 3- to 4 as susceptible. Where reaction types 2 and 2+ were recorded, the isolate was retested, and in most cases a susceptible reaction was recorded in the repeated tests.

Table I.1. Reaction Types.

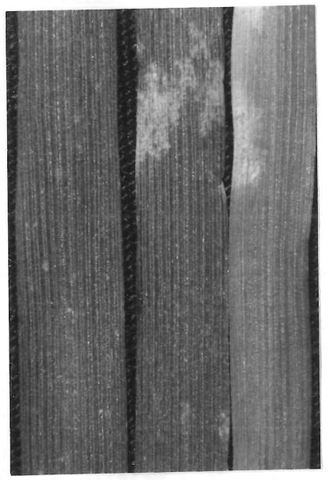
00	Hypersensitive reaction: numerous very small necrotic flecks.
0^n	Highly resistant: large areas of necrosis, distorting the shape of the leaf, chlorosis and no pustules.
0	Highly resistant: necrosis, chlorosis and no pustules.
1	Very resistant: necrosis, chlorosis and very small pustules.
2	Moderately resistant: chlorosis, less necrosis and small pustules.
3	Moderately susceptible: Large pustules, and little chlorosis.
4	Very susceptible: very large pustules, coalescent and no chlorosis.

The low incubation temperature of 7°C and high relative humidity necessary for germination, was seldom reached in the glasshouse, and this reduced the possibility of cross infection following the 24 hour incubation period in the refrigerator, and prevented spores of recent inoculations cross infecting the earlier inoculated material. Inoculations were carried out weekly. This meant that in the event of any cross contamination of earlier inoculated material, the contaminant isolates would have been 7 days later in developing than the test isolates.

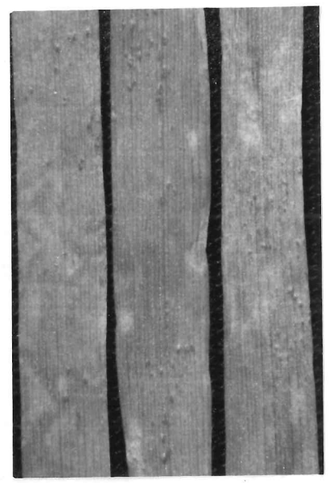
iv) Elimination of brown rust (P. recondita) contamination of rust stocks

During early experiments, before the installation of the isolation bench, contamination with brown rust was a serious problem. The two main reasons for this were: a low level of contamination in the first rust stocks used; and the ability of P. recondita to germinate at a higher temperature than P. striiformis which may have allowed spore germination to take place in the glasshouse overnight. P. recondita is faster growing

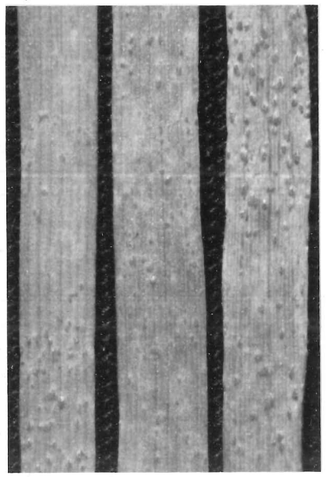
Plate I.3. Seedling reaction types.



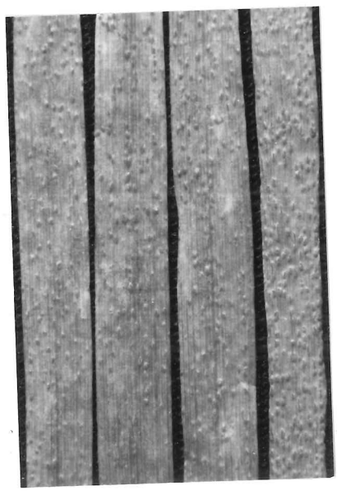
00 0 0^n



1- 1 1+

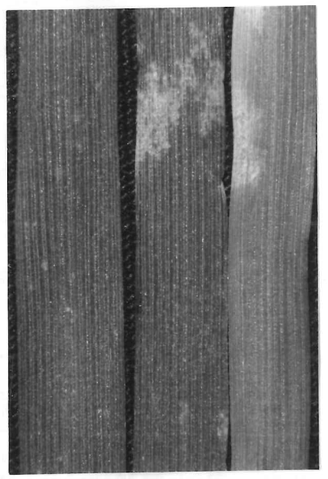


2- 2 2+

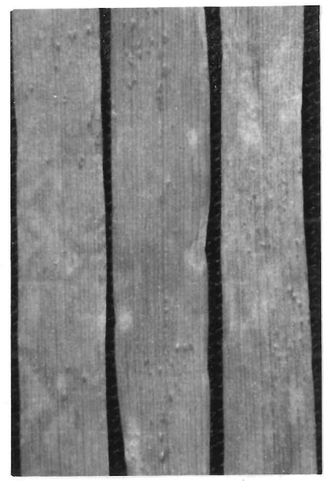


3- 3 3+ 4

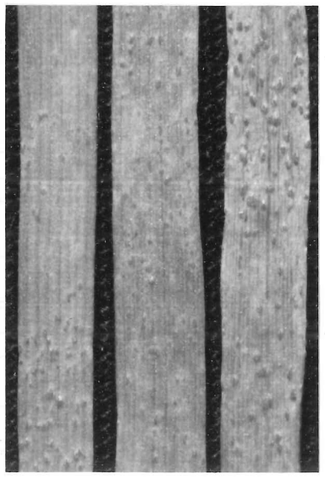
Plate I.3. Seedling reaction types.



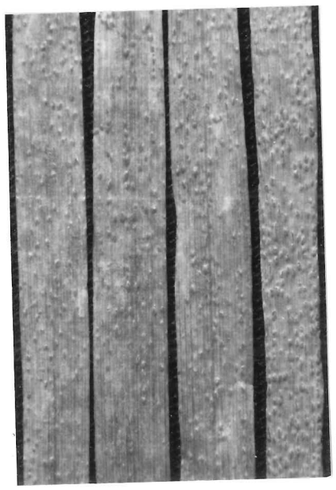
00 0 0^n



1- 1 1+



2- 2 2+



3- 3 3+ 4

than P. striiformis and the shorter generation time means that an infection of P. recondita will sporulate sooner, and may reduce the growth of P. striiformis, which does not grow as well as P. recondita in glasshouse conditions.

Contaminated yellow rust stocks were grown on the wheat variety PA 325 (Appendix I), which is resistant to P. recondita and susceptible to P. striiformis. The cultures were grown on this variety until there was no evidence of brown rust.

v) Differential varieties of wheat

In conjunction with the new race nomenclature, the new set of differential varieties have been used. Supplementary varieties were Heine 110 and Maris Envoy which are both susceptible to race 41 E 136 and resistant to race 104 E 137. Their reactions are therefore similar to those of Chinese 166 to these races, which was useful as the Chinese 166 seed stock was not consistently true in its reaction to race 41 E 136. Another supplementary variety, Maris Beacon, is resistant to race 41 E 136 and susceptible to race 104 E 137. The varieties Minister, Thatcher, and Cappelle-Desprez which contain the major genes for resistance Yr 3c, Yr 7, and Yr 3a and 4a respectively (Lupton and Macer, 1962; Macer, 1966b), were also included. Other known genes for resistance are found in the world and European sets of differential varieties: Chinese 166 (Yr 1), Heines VII (Yr 2), Hybrid 46 (Yr 3b and 4b), Triticum spelta alba (Yr 5), Heines Peko (Yr 6) and Compair (Yr 8) (Riley et al., 1968). Thus changes in virulence which would result in the ability to overcome any of these major genes could be detected.

A list of all the varieties used, and their reactions to races 104 E 137 and 41 E 136 are given in Table I.2.

Table I.2. Differential and additional varieties used in this work and the reaction to the two races 41 E 136 and 104 E 137, R = resistant, S = susceptible.

World Differentials	104 E 137	41 E 136
Triticum spelta var. album	R	R
Riebesel 47/51	R	R
Suwon 92 x Omar	S	R
Strubes Dickkopf	S	S
Moro	R	R
Vilmorin 23	S	S
Heines Kolben	R	R
Lee	R	R
Chinese 166	R	S
European Differentials		
Heines VII	S	S
Spaldings Prolific	R	R
Carstens V	R	R
Compair	R	R
Nord Desprez	S	S
Heines Peko	R	R
Reichersberg 42	R	R
Hybrid 46	S	R
Supplementary Varieties		
Cappelle-Desprez	S	S
Heine 110	R	S
Maris Beacon	S	R
Maris Envoy	R	S
Minister	S	S
Thatcher	R	R
Additional Varieties used		
Cama	S	S
Joss Cambier	S	S
Jubilegem	S	S
Maris Nimrod	S	S
Michigan Amber	S	S
PA 325	S	S
F_3 Hybrid 46 x Heine 110	R	R

All the necessary seed was available from the Plant Breeding Institute. The F_2 and F_3 stocks with homozygous resistance to the two races were selected from the cross Hybrid 46 and Heine 110.

vi) Development of special resistant lines

Some F_1 seeds of the cross Hybrid 46 x Heine 110 were used to produce F_2 families from which to select some plants exhibiting homozygous resistance to both races 41 E 136 and 104 E 137. These plants could then be used to test for any recombinant isolates.

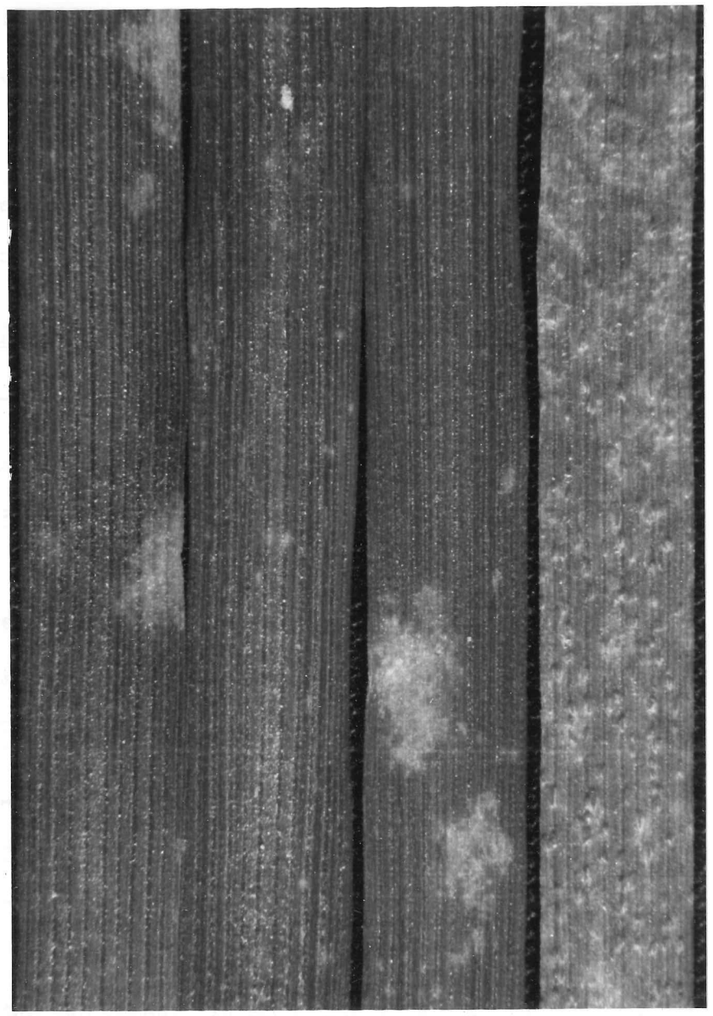
The F_1 seedlings were tested by inoculating the first leaf with race 104 E 137 and the second leaf with 41 E 136. The plants showed complete resistance to race 104 E 137, but gave an intermediate reaction to 41 E 136. This would suggest that resistance to race 104 E 137 is dominant, whereas resistance to race 41 E 136 is either incompletely dominant or controlled by more than one factor.

The F_2 seedlings were brush inoculated with both races on the first leaf. Race 104 E 137 was applied to the base of the leaf, and 41 E 136 was applied to the tip, leaving an uninoculated segment in the middle. Only plants which gave complete resistance to both races were selected. It was assumed that the observed resistance to race 41 E 136 was homozygous, but that resistance to 104 E 137 could have been homo- or heterozygous. Further testing of the F_3 populations was necessary in order to establish which lines were no longer segregating for resistance to 104 E 137.

One ear from each F_2 plant selected, was hand thrashed and the F_3 seeds were sown 5 or 6 to a pot, and tested with both races as for the F_2.

Plate I.4. Reaction types on F_3 selections of Hybrid 46 x Heine 110.

a = race 41 E 136
b = race 104 E 137
c = mixed races 41 E 136 and 104 E 137
d = race 105 E 137



a b c d

From the 35 single ears tested, it was evident that resistance to race 41 E 136 was not homozygous in all the lines, as in only 8 were all the plants resistant to it. Three lines gave complete resistance to both races, and these were selected. There was not enough seed available to use for routine testing, so the seed was only used for confirmatory tests on suspected recombinant isolates.

The F_3 lines selected gave a 00 type reaction to race 104 E 137, and a type 0 reaction to race 41 E 136. Both reaction types were observed when a mixture of both races was used as inoculum (Plate I.4).

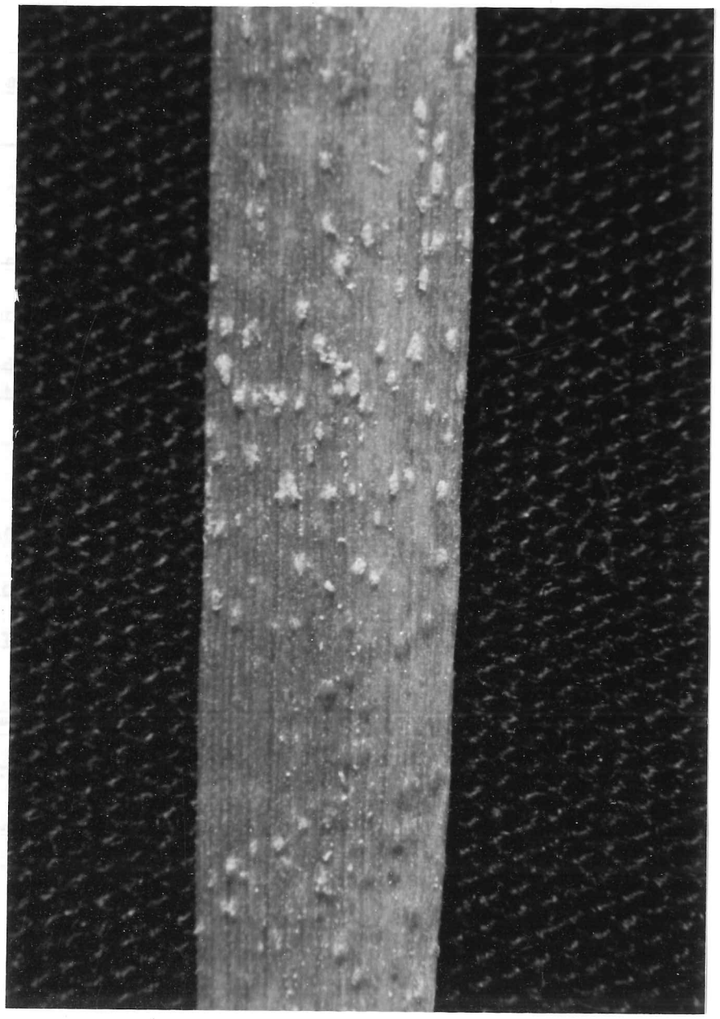
vii) Establishment of mixed race inoculations in order to obtain recombinant isolates

Seedlings of Heines Kolben, resistant to both race 104 E 137 and race 41 E 136, were inoculated with a spore-talc mixture of the two races in order to obtain recombinant isolates which had overcome this resistance. Despite three attempts, there was no sporulation on Heines Kolben, which consistently gave a type 0 or 0^n reaction. Therefore no single spore isolates were obtained from these inoculations.

Seedlings of Maris Beacon (susceptible to race 104 E 137, resistant to race 41 E 136), Maris Envoy (susceptible to race 41 E 136 and resistant to race 104 E 137), Maris Nimrod and Michigan Amber (both susceptible to races 41 E 136 and 104 E 137), were inoculated with a spore-talc mixture of the two races. In each case a type 4 reaction was observed, and the spores were collected. Single spore isolates were obtained from each mixed race infection (Table I.3).

Seedlings of Jubilegem, susceptible to both races, were inoculated with race 41 E 136 and the white mutant 104 E 137, in a spore-talc mixture.

Plate I.5. White and yellow rust mixed infection on Jubilegem.



Both white and yellow pustules developed (Plate I.5) and in some cases white and yellow spores were observed in the same pustules. Spores from these pustules were used to obtain 19 single spore isolates WR-1 to WR-19, which included some yellow isolates.

Table I.3. Host varieties and single spore isolates obtained from mixed race inoculations.

Variety	Number of isolates	Isolate reference numbers
Heines Kolben	None	-
Jubilegem	19	WR-1 to WR-19
Maris Beacon	10	MB-1 to MB-10
Maris Envoy	10	ME-1 to ME-10
Maris Nimrod	13	MN-1 to MN-13
Michigan Amber	7	MA-1 to MA- 7

Each of the single spore isolates obtained was multiplied up on the isolation bench, until sufficient spores were available for the tests outlined below.

The remaining spores from the mixed race inoculations on each variety, were kept and used for successive tests on the varieties Heine 110 and Hybrid 46, in order to screen the cultures for recombinant isolates.

viii) Establishment of single spore isolates

Single seeds of two varieties, Michigan Amber and Jubilegem, susceptible to both races 104 E 137 and 41 E 136 were sown in 5 cm pots in a spore free compartment. When the first leaves had fully expanded, groups of six pots were placed in each polythene bag, sealed, and taken to the laboratory.

Fine glass needles were prepared from lengths of glass tubing. Spores were placed on a watch glass under a binocular microscope, and the seedling

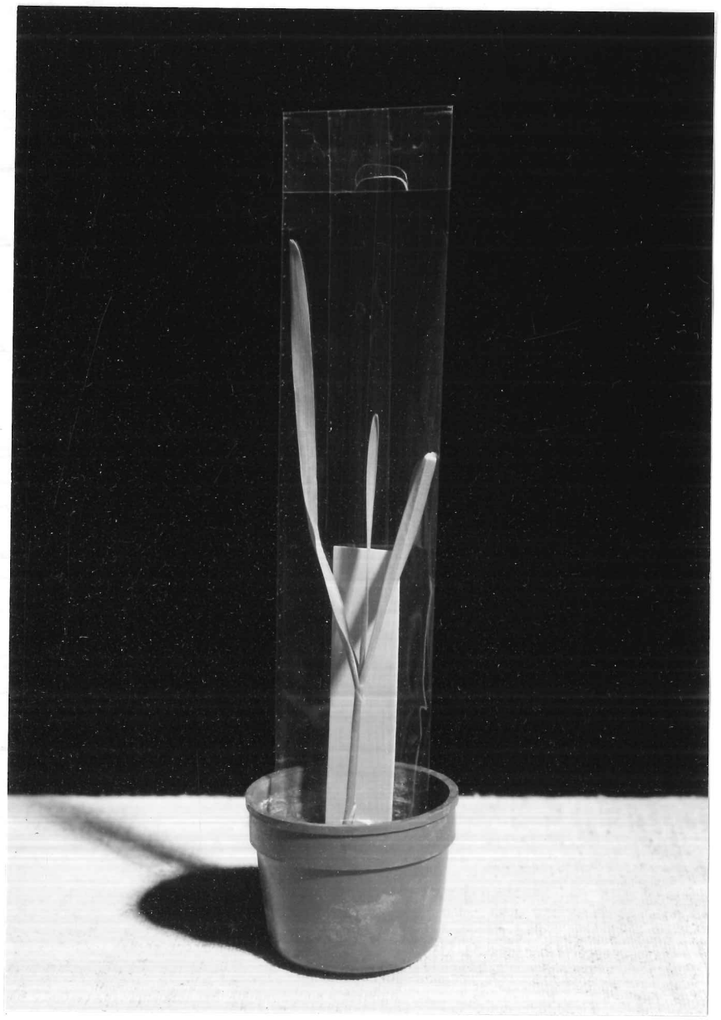


Plate I.6. Single spore isolate - 48 hrs after inoculation.
Cellophane cover to prevent cross contamination.

leaf of the plant was placed under another binocular microscope. A spore was picked up on the end of a glass needle, and carefully placed on the leaf surface. The needles were rinsed in alcohol and distilled water between each use.

When each of the six plants had been inoculated, they were placed in the polythene bag, sprayed with distilled water, and the polythene bag was then sealed. The bags of plants were placed in a refrigerator at 7°C overnight.

The following day, the plants were returned to the glasshouse, and each seedling was covered with a cellophane bag, supported by a label (Plate I.6). This prevented any cross contamination caused by other spores falling on the leaf, and avoided using the space on the isolation bench unnecessarily, as only about 7-15% of these single spore inoculations proved successful. At the onset of sporulation the infected plants were transferred to the isolation bench before any spores were collected. This meant that the spores which were collected had been released in the filtered air conditions of the isolation bench.

ix) Screening of mixed spore cultures

As only a very few spores were used in the establishment of single spore isolates, the remaining spores from the established mixed race infections were screened by successive inoculations of the varieties Hybrid 46 and Heine 110, in order to try and isolate any more virulent recombinants from the spore stocks.

Hybrid 46 (resistant to race 41 E 136) and Heine 110 (resistant to 104 E 137) were inoculated with spores from each infection. The plants were scored when the infections were fully established, and the spores were collected. The reciprocal inoculations were then established by inoculating seedlings of Hybrid 46 with spores from Heine 110 and vice versa.

x) Test inoculations of races and isolates

xa) Differential tests to identify races obtained from the mixed inoculations. Eight seedlings, of each of the differential varieties and supplementary varieties (Table I.2), were sown per pot in the spore free compartment. The seedlings were inoculated as described above and after the 24 hr refrigerated incubation period, the plants were returned to the test compartment. The pots were arranged in compact groups and the isolates were kept separate by a gap of about 15 cm.

Ten to twelve days after inoculation, the plants were sporulating. About 4 days later, when the reaction types were fully developed, the seedlings were scored, and the isolates were identified using the new race nomenclature (Johnson et al., 1972).

Two methods were used for assessing the overall reaction to the isolates: firstly the reaction types were converted to numerical values (Johnson et al., 1969) and the mean reaction type was then calculated; and secondly the number of reactions in each category, resistant, intermediate and susceptible were counted, and the category with the most values was taken as the varietal reaction to the isolate.

Both these methods gave the same results when the intermediate reactions had been individually considered, and the effects of temperature allowed for. Tests carried out during the optimal conditions (15-20°C) were given additional weighting. The reactions were thus classified into resistant and susceptible, and this made race identification possible.

xb) Experiments to establish the spore size of isolate MB-5. Initially spores were collected from single isolation bench pots of Jubilegem inoculated with either race 41 E 136 or isolate MB-5. The length

and width of 20 spores of each isolate were measured, and taking the spores to be regular ovoids, the spore volumes were calculated using the formula:

$$V = \frac{4}{3} \times ab^2$$

when a = half the length and b = half the width of the spore.

The preliminary results showed the two isolates to be significantly different, and a further experiment was conducted.

Nine isolation bench pots were sown with 20 seeds of Jubilegem per pot. Three pots were inoculated with one of each of the isolates MB-5, race 104 E 137 and race 41 E 136. The pots were placed on the isolation bench in a randomised block design.

When the plants were sporulating, the spores from each pot were collected, and as some of the difference in spore size in the preliminary experiment could have been due to differences in hydration of the spores from the different pots, the spores were tapped on to 1% agar plates. The plates were left for 18 hours to ensure that all the spores were similarly hydrated, and the dimensions of twelve spores from each plate were measured. The spore volume was calculated and the results analysed.

xc) Experiments to establish the generation time of isolates.

Most of the single spore isolates were found to be races 41 E 136 and 104 E 137. It was felt that some useful information on differences within races might be obtained if the time between infection and sporulation was studied. The single spore isolates could be regarded as samples of the fungal population, which could give some indication of variability within the races 41 E 136 and 104 E 137, although each group came from a single pure culture.

In each of 11 experiments, numbered 1 to 11, four single spore isolates, referred to by isolate numbers, and the two 'parental' races 41 E 136 and 104 E 137 were compared. Two isolates obtained from field plots of Joss Cambier, 71/2 (race 104 E 137) and 72/40 (race 41 E 136) were included in experiment 6.

Four winter wheat varieties were chosen: Heine VII and Joss Cambier, susceptible to both the races; Hybrid 46, resistant to 41 E 136; and Heine 110, resistant to 104 E 137. Thirty pots of each variety were sown with five seeds per pot. When the seedling leaves were fully expanded, five pots of each variety were placed in each inoculation chamber, and the seedlings were inoculated using one of the four test isolates or race 41 E 136 or race 104 E 137. The spores used were less than one week old, and had not been freeze dried.

Following the incubation period, the plants were returned to the test compartment, and arranged in five randomised blocks, each block containing one of each of the 24 treatments.

The seedlings were examined daily and at the first signs of infection the seedlings were tagged. The date of flecking and sporulation was recorded. Sporulation was considered to have started when the pustules had first ruptured the host's epidermis, as it was possible to observe this accurately; the timing of any later stage, for example presence of free spores, would have been less precisely estimated and would therefore have given a larger experimental error. The seedling reaction types were recorded 16 days after inoculation, by which time, in all cases observed, the reaction types were well developed.

The results for each pot were totalled, and the incubation period (period from inoculation to signs of infection), latent period (period from inoculation to sporulation), and lag period (period from visible symptoms to sporulation) were analysed, together with the seedling reaction. The latent periods of the resistant varieties were analysed as missing plots, as it was not possible to establish when the resistant reaction was fully developed, or when it had reached a stage comparable to the spores rupturing the epidermis in the susceptible reactions.

In order to check whether spore viability, assessed as percentage germination and penetration frequency, of the isolates was affecting the generation time, thirteen isolates which showed distinctly faster or slower development than the 'parental' isolates were used to inoculate isolation bench pots of Jubilegem. The spores of each isolate were then used to inoculate single plants of the four varieties. The inoculated seedlings were returned to the glasshouse and placed in four randomised blocks. The seedlings were harvested after 5 days by which time the germ tubes should have penetrated the epidermis, and the penetration points were well developed.

The leaves were boiled and stored in alcoholic lactophenol and were transferred to alcoholic lactophenol cotton blue (Shipton and Brown, 1962) 24 hrs before examination. Approximately 1 cm of the leaf, taken 1 cm from the tip, was scanned and the number of penetration points in the segments was established.

Each isolate was also used to inoculate 8 plates containing 1% agar which were incubated at 7°C overnight to obtain germination estimates. The plates were fixed immediately following the incubation, using alcoholic lactophenol cotton blue.

I3. Experimental Results.

i) Screening of mixed spore cultures

a) When Heine 110 was inoculated with spores from the mixed race infection on Maris Beacon, a very high number of resistant seedlings were obtained. A few susceptible seedlings were observed with reaction types 3 or higher. However on Hybrid 46, all the seedlings were susceptible.

In the reciprocal inoculations, the spores off Heine 110 gave 3- to 4 type reactions on Heine 110, but all the reaction types were observed on Hybrid 46, and half of the seedlings were classified as susceptible. The spores from Hybrid 46 gave 00 reactions on Heine 110 and 3 to 4 reactions on Hybrid 46.

b) Seedlings of Hybrid 46 which had been inoculated with the spores from the mixed infection on Maris Envoy gave predominantly resistant reactions. Although about half of the seedlings showed some evidence of sporulation, only about 15% were classified as susceptible. On Heine 110 however, no reactions lower than 3+ were observed.

Surprisingly, the reciprocal inoculations of spores collected off Hybrid 46 gave a uniform infection of type 3+ and 4 reactions on Heine 110, but illustrated a variety of reactions on Hybrid 46 of which only just over half were susceptible. The spores collected from Heine 110 gave largely resistant reactions on Hybrid 46. Although a low level of sporulation was observed on half the seedlings, only about 1% were susceptible. On Heine 110 the situation was reversed, and about 1% of the plants gave resistant reactions, in contrast to the remainder, which were type 4.

c) Both Hybrid 46 and Heine 110 gave susceptible reactions when inoculated with the mixed race infection spores from Maris Nimrod. About 17% of the Heine 110 seedlings gave a type 2 or lower reaction and were classified as resistant, whereas the Hybrid 46 seedlings were all fully susceptible.

When Hybrid 46 was inoculated with spores collected from Heine 110, 62% of the plants gave some evidence of sporulation, but only 17% could be classified as susceptible. When spores from the Hybrid 46 collection were used, all the seedlings were susceptible and no scores below 3 were recorded. Heine 110, when inoculated with spores from Hybrid 46 gave predominantly resistant reactions, with 19% of the seedlings giving type 3+ and 4 susceptible reactions, as point infections. When spores from Heine 110 were used, the reactions were all type 4.

d) When Heine 110 was inoculated with the mixed spores from Michigan Amber, only 16.5% of the seedlings were susceptible, but 98% of Hybrid 46 seedlings were susceptible when inoculated with the same spores.

When the spores collected from Hybrid 46 were used to infect Heine 110, complete resistance was observed in all the seedlings, but seedlings of Hybrid 46 gave type 3+ to 4 throughout. The spores collected from Heine 110 gave fully susceptible reactions on all seedlings of Heine 110 but surprisingly only 5% of the Hybrid 46 seedlings were resistant.

e) Spores from the white and yellow mixed race inoculations gave good infections of yellow pustules on Heine 110 and white pustules on Hybrid 46. There were no yellow pustules on Hybrid 46 and no white pustules on Heine 110. The same was observed in the reciprocal inoculations.

The seedling reactions from the first and second inoculations are summarised in Table I.4 and Table I.5.

Single spore isolates were established from the infections marked * in Table I.5 and their isolate numbers are given in Table I.6. Attempts to establish single spore isolates from the spores collected off Maris Nimrod were not successful, despite three separate attempts.

Table I.4. First inoculations of spores from mixed race infections on Hybrid 46 and Heine 110. Percentage of susceptible seedlings observed

Mixed infection on variety		Test Varieties	
		Heine 110	Hybrid 46
Maris Beacon	yellow isolates used	10.6	100.0
Maris Envoy	yellow isolates used	100.0	15.6
Maris Nimrod	yellow isolates used	83.0	100.0
Michigan Amber	yellow isolates used	11.5	97.6
Jubilegem	(white and yellow isolates used)	74.0	98.0

Table I.5. Second inoculations of spores derived from mixed race infections on to Hybrid 46 and Heine 110. Percentage of susceptible seedlings observed

Mixed infection on variety	First Test variety	Second Test variety	
		Heine 110	Hybrid 46
Maris Beacon	Heine 110	100.0	50.0*
	Hybrid 46	0.0	100.0
Maris Envoy	Heine 110	99.0	1.0
	Hybrid 46	100.0*	50.0
Maris Nimrod	Heine 110	100.0	17.0
	Hybrid 46	19.0	100.0
Michigan Amber	Heine 110	100.0	95.0*
	Hybrid 46	0.0	100.0
Jubilegem	Heine 110	100.0	0.0
	Hybrid 46	0.0	100.0

Table I.6. Single spore isolates obtained from infections marked * in Table I.5 and isolate numbers

Host variety	Number of isolates	Isolate Reference Number
Maris Beacon	2	MB-11 to MB-12
Maris Envoy	4	ME-11 to ME-14
Michigan Amber	4	MA- 8 to MA-11

ii) Differential tests to identify races obtained from the mixed race inoculations

The temperature in the glasshouse varied with the season, but all the tests were carried out between mid-March and October, during which the average temperatures ranged from 17° to 30°C. Daylight intensity increased during the hotter weather, and this evidently helped to ensure that seedling reactions were comparable between tests. Two varieties Carstens V and Spaldings Prolific, normally resistant to both races were more susceptible during the hot weather and sometimes gave type 4 reactions but isolates which were re-tested in cooler temperatures illustrated the resistant reactions on both varieties. None of the isolates gave consistently susceptible reactions on these varieties.

It was noticed that seedlings from individual plants of the differential variety Chinese 166 showed two extremes of reaction types with race 41 E 136. Several seed stocks were tested simultaneously with race 41 E 136 and 104 E 137 on all the seed stocks, but two distinct reaction types were observed using race 41 E 136, 0-1 and 3-4. The 0-1 and 00 reactions were different enough to distinguish between the races and for this reason the 0-1 reactions on Chinese 166 were classified as a susceptible reaction.

This was supported by the reaction on Heine 110, the supplementary variety added to overcome this problem.

Of the 50 yellow single spore isolates tested, 39 proved to be the same as the parental races: 12 were race 41 E 136, and 27 were race 104 E 137. Eleven isolates were suspected recombinants, as they gave the race number 105 E 137 from the differential tests.

The suspected isolates were tested on the selected F_3 Hybrid 46 x Heine 110 seedlings. Eight of the isolates gave mixed type 0 and 00 reactions, and were found to be mixed races. Three isolates, MB-5, MB-6 and MN-7, gave type 4 reactions, which illustrated the recombined virulence of 41 E 136 and 104 E 137, and confirmed the race number 105 E 137. This race had not previously been recorded.

Infections of isolate MB-5 appeared to give rise to larger and more distinct pustules than did MB-6 or MN-7, which were indistinguishable in appearance from the parental race infections. It was felt that MB-5 might have larger spores than race 41 E 136 or 104 E 137, and this was tested in a separate experiment.

A test using ME-4 gave an isolated infection point on a seedling of Heines Kolben, a variety resistant to both races, and on which no infection had been obtained in mixed race inoculations. Spores from this infection point were collected and multiplied on pots of Jubilegem on the isolation bench. The isolate, identified as ME-4(i), was retested and identified as race 37 E 132 (race 60). This isolate was picked up in May 1973, and although no work on this race had been carried out in the glasshouse during the preceeding 4 months, it is likely that it was a contaminant.

Of the nineteen isolates obtained from the mixed yellow and white races, seventeen were white and two were yellow. When tested, all the 17 white isolates were found to be race 104 E 137, and both the yellow isolates were race 41 E 136.

The number of single spore isolates of each race obtained are given in Table I.7.

Table I.7. Numbers of single spore isolates of each race obtained

Host variety	Mixed isolates & 'others'	104 E 137 isolates	105 E 137 recombinant isolates	41 E 136 isolates
Jubilegem	0	17*	0	2
Maris Beacon	1	9	2	0
Maris Envoy	6	1	0	8
Maris Nimrod	1	7	1	4
Michigan Amber	0	11	0	0

* White isolates specifically chosen.

iii) Differences in spore volume between MB-5 and races 41 E 136 and 104 E 137

From the initial experiment, the mean spore volumes recorded were 5.96×10^3 μm^3 for race 41 E 136, and 9.38×10^3 μm^3 for isolate MB-5. The difference was significant at the 0.1% level.

The hydrated spores in the second experiment were larger and there was a smaller difference between mean spore sizes. The distribution of spore volumes recorded is illustrated in Fig. I.1 and the mean spore volumes are given in Table I.8.

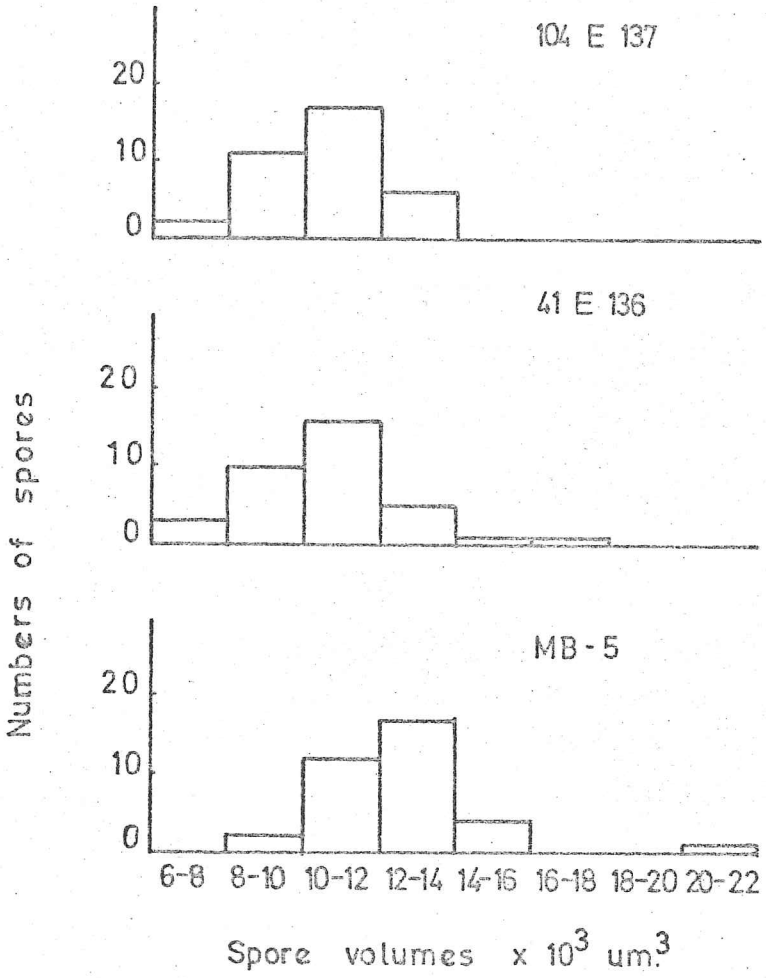


Fig I.1 Distribution of spore volumes.

Table I.8. Mean hydrated spore volumes of races 41 E 136, 104 E 137, and isolate MB-5

Race/Isolate	Spore volume ($\times 10^3$ μm^3)
104 E 137	10.27
41 E 136	10.54
MB-5	12.60

Standard error = 1.27; P = 0.001

The two 'parental' races do not differ significantly from each other, but the isolate MB-5 is significantly larger than both 'parental' races. The nuclei of this isolate were also studied, and were found to be no larger than the nuclei of the 'parental' races. The number of nuclei also did not differ from the usual 2.

iv) Experiments to establish the generation time of isolates

A few isolates showed signs of flecking by 4 days after inoculation on isolated seedlings, but more often they did not show any flecking before 5 days. In the majority of cases, flecking was observed between 5 and 7 days from inoculation, although in some cases 9 and even 10 days passed before flecking was observed.

Races 41 E 136 and 104 E 137 did not have significantly different incubation periods, while isolates MA-1 and ME-13 were both faster than the two 'parental' races, and isolates ME-4, MB-4, MB-5, MN-2, MN-6, MN-11 and 72/40 were all slower.

Sporulation occurred between 8 and 15 days from inoculation, but 9 to 11 days were the most frequently recorded. Two isolates, MA-1 and MB-2 were both faster than the 'parental' races up to sporulation, and

8 isolates were slower: MB-4; MB-9; MB-10; MN-1; MN-2; MN-3; MN-6 and 72/40.

The lag period between flecking and sporulation ranged from 2 to 8 days, and 4-5 days was the most common period. MA-1 again showed a shorter lag period than did the 'parental' races, as did MN-7, which was not significantly different from the parental races at flecking or sporulation.

Isolates MB-4, MB-9, MB-10, ME-10, MN-1, MN-2, MN-3, ME-7 and 72/40 all gave a longer lag time than the 'parental' races. Of these isolates, MB-4, MN-2 and 72/40 were consistently slower at each stage than the 'parental' races, and isolates ME-10 and MN-7 gave a longer lag time despite not being significantly different than the 'parental' races at flecking and sporulation.

The mean reactions of each isolate on each variety were plotted against the incubation and latent period times observed. Only observed values were used, which meant that the latent period values for resistant varieties were excluded as these were calculated from missing plot figures. As the reaction types were not recorded in the first of these experiments, no data were available for this experiment.

A negative linear regression ($p = 0.012$ to $p < 0.01$) was observed for the latent period in all but two of the experiments, (Fig. I.2). Of the 8 significant regressions the regression lines of 6 experiments cut the y axis at x=0 between 12.30 ± 0.34 and 13.42 ± 0.78. The other two values obtained were 11.18 ± 0.20 in experiment 3 and 20.07 ± 4.71 in experiment 2. The value obtained for experiment 2 was probably caused by the cooler temperatures during April, but all these results show that an increase in the level of resistance exhibited by the host results in

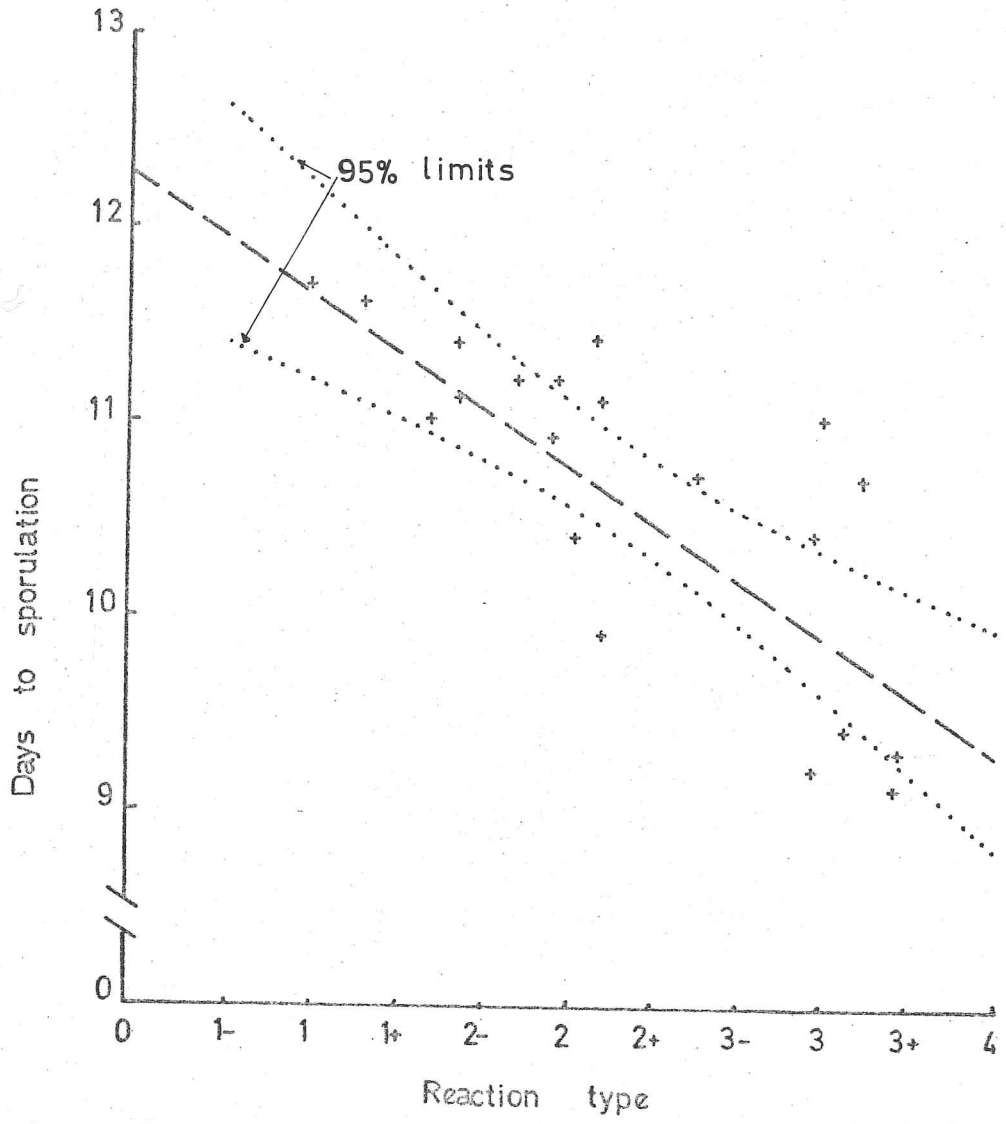


Fig I.2 Influence of reaction type on latent period.
b = -0.294 $p < 0.01$

an increased latent period, although the incubation period did not show a direct correlation.

The data obtained for incubation and latent period generally fell into a normal distribution (Fig. I.3.). The modes for flecking and sporulation are given in Table I.9.

Table I.9. Most frequent period observed between inoculation and flecking and inoculation and sporulation

Experiment	Incubation period	Latent period
1	7	12
2	6	11
3	6	10
4	5	9 and 11*
5	5	10
6	5-6	9 and 11*
7	5	9
8	5	9
9	5	10
10	5	9
11	5	9-10

* Distinct double peak

The variation in the peak onset of flecking and sporulation were probably due to the glasshouse temperature, as the earliest experiments were carried out in March-April and subsequent experiments were carried out through the summer months until September-October.

In experiments 4 and 6 two distinct double peaks were observed in the latent period data, although not evident in the incubation period data. The isolates in experiment 4 were two of the suspected recombinant isolates ME-4 and MB-3. Each isolate had been collected off both Hybrid 46 and Heine 110, thus creating four subisolates: these were found to be mixed race cultures and not new races, and would account for the longer incubation period.

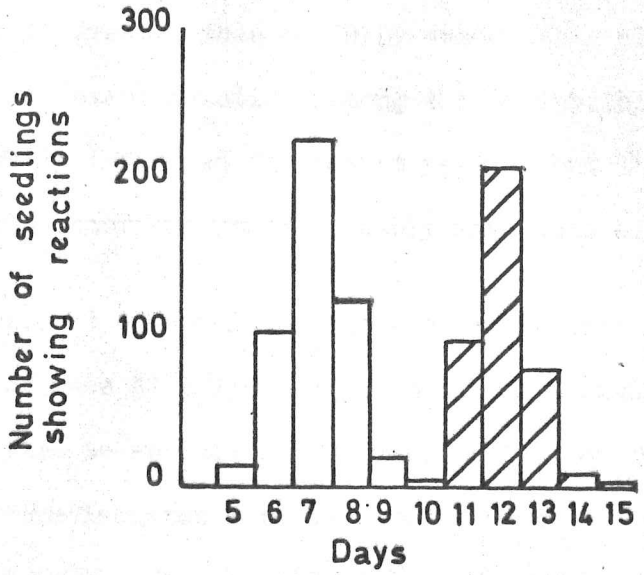


Fig I, 3 Development of infections in Experiment I.

□ fleck ▨ sporulation

A few spores of race 41 E 136 were present in the isolate collections off Hybrid 46 (largely race 104 E 137) and a few spores of race 104 E 137, in the collection off Heine 110 (largely 41 E 136). These spores would have been competing with the resistant reactions established by the majority of the infection points created by the 104 E 137 and 41 E 136 spores on Heine 110 and Hybrid 46 respectively.

As outlined above, the latent period of intermediate and resistant reactions is greater than on fully susceptible plants. The presence of a few resistant reactions among the susceptible seedling could also have slightly increased the latent period, but the frequency of susceptible reactions would probably mask this effect.

Experiment 6 was one of the two experiments in which the parental isolates of race 41 E 136 and 104 E 137 were significantly different in their time to sporulation, race 41 E 136 being the faster race. In this case the isolates were compared with their 'parental' race. MA-1 was significantly faster, and MN-12 although not quite significant at the 5% level, was slower than the 'parental' race 104 E 137. Isolate 72/40 was significantly slower than 41 E 136. These distinctly varying isolate times would therefore account for the double peak.

The percentage germination on agar obtained from the 13 isolates tested showed significant differences between isolates, ($p < 0.01$). Race 104 E 137 had a higher level of germination, 66.25%, than did race 41 E 136, 43.0%. Four isolates differed significantly from each of the parental isolates.

The number of penetration points per sq. mm of leaf surface also showed a significant difference between isolates ($p = 0.001$), and race 104 E 137 had more penetration points per sq. mm (1.52), than did 41 E 136 (0.52). However, there was no direct correlation between spore

germination and penetration frequency. Four 104 E 137 type isolates differed from the 'parental' race and all had fewer penetration points, although two of the isolates, MB-2 and MA-1 gave faster development than race 104 E 137. One isolate of 41 E 136 had significantly more penetrations per sq. mm (1.10) than did the parental race, and this isolate was also slower than 41 E 136 in the generation time experiments.

From these results, it would seem that isolate generation time was independent of both spore germination and penetration frequency.

Some indication of the effect of temperature on the rate of rust development can be obtained from comparisons of some data from 4 experiments in which the mean maximum day and mean minimum night temperatures were as follows: 17.1° and 10.0°C; 21.7° and 11.9°C; 26.0° and 12.6°C; and 29.0° and 14.9°C. The experiments were carried out during March through to June, and the mean Incubation, Latent and Lag periods for the two races are given in Table I.10.

Table I.10. Mean incubation, latent and lag periods in days observed for races 41 E 136 and 104 E 137 at four different temperature regimes

Stage	Temperature Regimes: °C			
	17.1 & 10.0	21.7 & 11.9	26.0 & 12.6	29.0 & 14.9
Incubation Period				
41 E 136	6.8	5.8	5.8	5.4
104 E 137	6.5	5.8	5.8	5.3
Latent Period				
41 E 136	12.0	10.6	9.8	9.8
104 E 137	11.1	10.2	9.6	9.3
Lag Period				
41 E 136	5.0	4.7	4.0	4.3
104 E 137	4.6	4.4	3.8	4.2

I4. Discussion

The formation and isolation of race 105 E 137, a new race which combines the virulence of race 104 E 137 and 41 E 136, can best be considered in relation to the races of major importance in Britain at the time of writing, and a newly identified race* 45 E 140. The 4 races of major importance in the British Isles at the time of writing are 37 E 132 (race 60), 41 E 136 (race 58C), 104 E 137 (race 3/55D) and 108 E 9 (race 1B). In Table I.11 the reactions of each race on the differential varieties are given. The likely reaction which would be obtained in the event of recombination between two races are given in Table I.12.

Of the three proposed recombinant isolates, 45 E 140 has been provisionally identified by the National Institute of Agricultural Botany (N.I.A.B.) and 105 E 137 has been isolated from the work outlined above. In each case the new race number is obtained from the addition of two races prevalent in this country at present.

Race 105 E 137, produced during controlled experiments in the glasshouse, illustrates the possibility of such new race formation being able to take place when two races are grown in close proximity. The isolation of race 45 E 140 must surely be evidence that the occurrence of such recombination is not confined solely to controlled glasshouse experiments. This should therefore cause some apprehension when plant breeders test new varieties in the field with artificial mixed race infections, however the risks and advantages must be counterbalanced.

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* Provisionally identified by the NIAB physiologic race survey. My thanks to Drs. J.K. Doodson and R.H. Priestley for making this information available to me.

Table I.11. Reactions of the differential varieties to four major races of Puccinia striiformis

World Differentials	No.	Race 60 37 E 132	Race 58C 41 E 136	Race 3/55D 104 E 137	Race 1B 108 E 9
Triticum spelta var. album	256				
Riebesel 47/51	128				
Suwon 92 x Omar	64			*	*
Strubes Dickkopf	32	*	*	*	*
Moro	16				
Vilmorin 23	8		*	*	*
Heines Kolben	4	*			*
Lee	2				
Chinese 166	1	*	*		
European Differentials					
Heines VII	128	*	*	*	
Spaldings Prolific	64				
Carstens V	32				
Compair	16				
Nord Desprez	8		*	*	*
Heines Peko	4	*			
Reichersberg 42	2				
Hybrid 46	1			*	*

Table I.12. Likely reactions which would be obtained in the event of a direct recombination of any two of the races above

		Race recombinations:					
World Differentials	No.	60 & 58C	60 & 3/55D	60 & 1B	58C & 3/55D	58C & 1B	3/55D & 1B
Triticum spelta var. album	256						
Riebesel 47/51	128						
Suwon 92 x Omar	64		*	*	*	*	*
Strubes Dickkopf	32	*	*	*	*	*	*
Moro	16						
Vilmorin 23	8	*	*	*	*	*	*
Heines Kolben	4	*	*	*		*	*
Lee	2						
Chinese 166	1	*	*	*	*	*	
World Number		45	109	109	105	109	108
European Differentials							
Heines VII	128	*	*	*	*	*	*
Spaldings Prolific	64						
Carstens V	32						
Compair	16						
Nord Desprez	8	*	*	*	*	*	*
Heines Peko	4	*	*	*			
Reichersberg 42	2						
Hybrid 46	1		*	*	*	*	*
European Number		140	141	141	137	137	137

From the mixed race inoculations of yellow isolates, 50 single spore isolates were obtained and 3 of these were the recombinant race 105 E 137, which gives a recombination rate of 6%; although lower than the 10% obtained by Little (1966) with races 8B (32 E 128) and 2B (40 E 72), this is still a relatively high recombination rate. This would also suggest that the race 105 E 137 had a competitive advantage over the 'parental' races on Maris Nimrod, and also that the isolates obtained from Maris Beacon and Maris Envoy were as competitive as the virulent race on each of the host varieties.

Races 104 E 137 and 41 E 136 differ in their reactions on 3 of the differential varieties, Suwon 92 x Omar, Hybrid 46 and Chinese 166. The loss of virulence of race 41 E 136 on Chinese 166 or of 104 E 137 on Suwon 92 x Omar and Hybrid 46, which react similarly to all races, would result in 40 E 136 (3/55 Cleo). Race 40 E 136 has not been detected in the single spore isolates, which would suggest, assuming the recombination which resulted in the loss of virulence had occurred, that the recombinant was less well adapted to the host varieties and was unable to compete favourably with the other races on the host varieties. This would certainly be true on Hybrid 46 and Heine 110, as race 40 E 136 is avirulent on these varieties. Race 40 E 136 is virulent on both Michigan Amber and Maris Nimrod, so its formation on these varieties should have been possible.

The fact that race 40 E 136 was not obtained from this work does not however preclude the possibility of its formation: race 40 E 136 would not be detected during the bulk spore screening on Hybrid 46 and Heine 110, as it is avirulent on both these varieties; single spore isolates are difficult to establish (less than 2% of all attempts were successful) and only very few of the spores produced are sampled in this way. This

therefore elucidates the problems of isolating races with a narrower virulence spectrum than the 'parental' races in this type of work.

The new race 105 E 137 could have arisen by any one of four asexual methods: Mutation; Extrachromosomal inheritance; reassortment of whole nuclei; and the parasexual cycle. The parasexual cycle will be discussed in the light of the spore size results.

Spontaneous mutation in pathogenicity has been evident in the rusts, in P. graminis tritici (Watson, 1957a), P. coronata avenae (Zimmer et al., 1963) and more recently in P. striiformis (Chamberlain et al., 1970). Watson (1957a) however, pointed out from his glasshouse studies that it was not possible to say whether the isolates were genuine mutations, or whether 2 or more genes were involved. Chamberlain et al. (1970) however, suggested that a single step mutation from race 3/55 Cleo on Chinese 166 could have given rise to the new virulence combination of race 58C. In both cases the new combined virulence could not be attributed to the recombination of known virulence factors of other known races.

The recombinant isolates obtained in this work however show the combined virulence of the races 41 E 136 and 104 E 137 used in this work. Both the 'parental' races remained true in their reactions on the differential varieties, and yet the recombinant isolates were obtained from mixed inoculations on two varieties, Maris Beacon and Maris Nimrod. It is possible that both the isolates from Maris Beacon arose from the same colony, but even two recombinants out of 50 single spore isolate cultures established, would reflect a high mutation rate. The mutation rates in rust seem to be low (Stakman and Harrar, 1957) and the high frequency with which these new race 105 E 137 isolates were obtained would cause considerable doubt that mutation was the mechanism by which they arose.

The white isolate obtained from glasshouse work by others in the pathology section was probably due to mutation. Precise data on mutation rates in the rust fungi are not available. This white isolate arose from a single infection point on an inoculation of race 104 E 137. The mutation was solely confined to spore colour, and the virulence spectrum was the same as the race from which it was isolated. Only one other white mutant has been reported (Brown and Sharp, 1970), and the infrequent occurrence could be indicative of a low natural mutation rate of P. striiformis for this character.

Although the white isolate showed equal vigour in the mixed race infection of Jubilegem, no recombinant white-yellow isolates were obtained among the single spore isolates, or from the bulk screening tests. The purity of the reactions in each of the inoculations during the screening is of interest, as in the mixed yellow race cultures, some level of contamination was observed both in the first and second test inoculations, which would suggest that a few spores of the avirulent race were able to become established once the virulent race had overcome or bypassed the plant's resistance. This was not the case with the mixed white and yellow inoculations, as at no time in these tests were white pustules obtained on Heine 110 or yellow on Hybrid 46.

Yellow and white spores were observed arising from the same pustules (Plate I.5) and in taking single spore isolates, spores from such pustules were used where possible. Some of the spores produced seemed to have a pale beige colouring, possibly an intermediate between white and yellow. Isolates obtained from these spores however were no different in virulence from the white ones and were all race 104 E 137.

Nineteen isolates is a small number, and one cannot say from these results that spore colour is race linked, or that recombination does not take place. It is possible that more generations in a mixed race population might increase the likelihood of obtaining recombinants, and a larger number of isolates obtained would greatly increase the chance of locating and identifying any such recombinants if they arise. This, however, was not possible here, as the white isolate was not found until the experimental work was nearing completion.

Experimental evidence favouring the operation of extrachromosomal inheritance is difficult to explain. Johnson et al. (1934) have shown that virulence can be controlled by such particles in P. graminis tritici, and Jinks (1957 and 1959a) has shown that in Aspergillus glaucus variation in characters such as spore germination, growth rate, and pigmentation are under cytoplasmic control during differentiation and ageing. Jinks (1959b) also pointed out that many features of the 'dual phenomenon' in imperfect fungi which have been explained in terms of the segregations of a heterokaryon into its homokaryon components can be explained in terms of cytoplasmic variation.

Although cytoplasmic inheritance is best illustrated by using reciprocal crosses, Jinks (1954) outlined a method by which imperfect fungi can be tested without the use of reciprocal crosses. However, it has not been possible to use this method in this work as P. striiformis cannot be maintained in artificial culture, and for this reason, while one cannot totally exclude the presence of extranuclear inheritance, there is no direct evidence favouring this method, although the work on generation time below could indicate some level of cytoplasmic influence of isolates, and explain some of the variation within races.

Nelson et al. (1955) have demonstrated the occurrence of whole nuclear reassortment in connection with the development of new rust races. They mixed races 38 and 56 of P. graminis tritici and obtained a virulent trinucleate biotype which was able to attack the variety Khapli, which was resistant to both the original races. The biotype was however unstable, and with time it dissociated, providing two new biotypes. Little and Manners (1969a) found that whole nuclear reassortment seemed the most likely method to explain the formation of two new race types from races 2B and 8B (40 E 8 and 32 E 160) of P. striiformis, and Konovalova and Schekotkova (1970) produced an unstable 'heterokaryon' with reduced virulence on most of the differential varieties, but which was able to attack Cappelle Desprez, which the original races used were unable to attack. The isolate remained stable for 3 generations, after which it reverted to its original form.

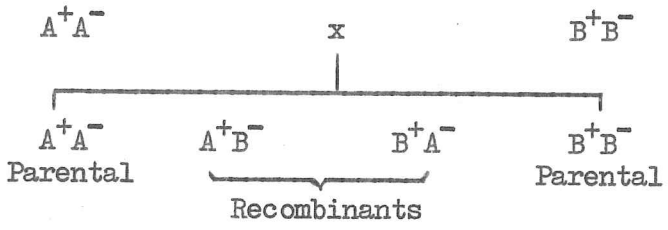
If one considers the possibility of nuclear reassortment being operational, two factors need consideration:

i) Isolate MB-5 had a significantly larger spore size than did the two original races. Is it therefore a trinucleate as in the Khapli infecting biotype?

ii) Only one virulence recombination was obtained among the progeny, and a maximum of two stable new races would be expected if the fungus is heterothallic and up to four if the fungus was homothallic.

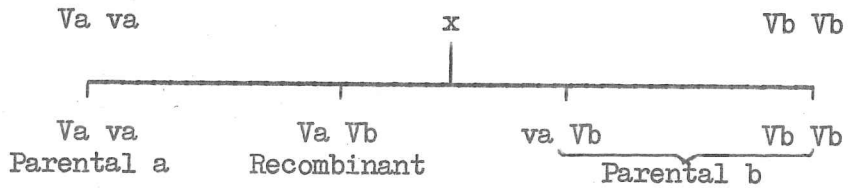
Cytological studies failed to provide any evidence to suggest that isolate MB-5 was a trinucleate biotype. The isolate remained stable in its reaction throughout the work.

Although only one new combination of virulence was obtained this would not necessarily eliminate the possibility of others being formed. It is possible that another, less virulent biotype was formed. Buller (1950) established that most of the rusts were heterothallic, and it is likely (Little and Manners, 1969a) that P. striiformis is also heterothallic. If this is the case, the production of the new races by reassortment of nuclei can be represented diagrammatically below:



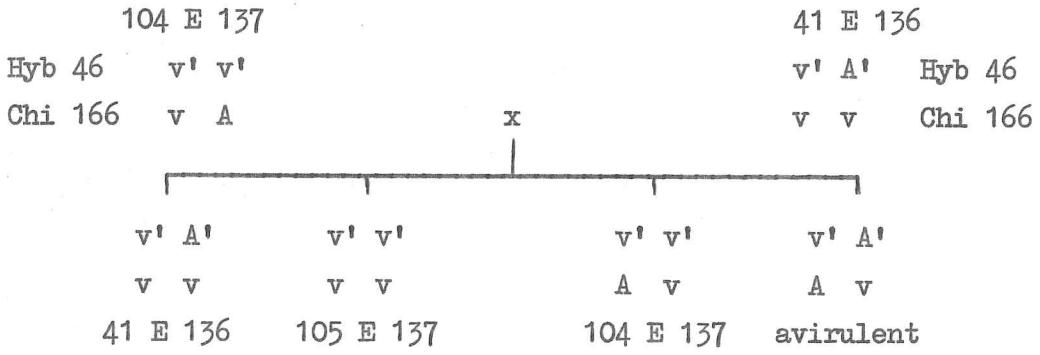
If the nuclei are heterozygous for virulence on Hybrid 46 and Chinese 166 and A^+ and B^- contain dominant factors for virulence, the recombinant A^+B^- would represent race 105 E 137, and B^+A^- would represent the avirulent isolate not successfully isolated.

If one race was homozygous and the other was heterozygous the isolates would arise as follows:



This would mean that one race would be present at a higher frequency than the other, and if virulence on Hybrid 46 is homozygous, this would account for the more frequent detection of race 104 E 137 than race 41 E 136. If both races were homozygous the avirulent race would not be formed.

If virulence is recessive, and race 104 E 137 is heterozygous for virulence on Chinese 166 while 41 E 136 is heterozygous for virulence on Hybrid 46, the situation can be represented as follows:



This would result in only one 'new' combination using Hybrid 46 and Chinese 166 or Heine 110 as differentials. This would seem a likely explanation as virulence is more often reported as recessive than as dominant.

Recombinant isolate MB-5 showed a significantly larger spore size than did the races 41 E 136 and 104 E 137. In the first experiment the fresh spores were tapped on to 1% agar and measured immediately - isolate MB-5 was measured before race 41 E 136, so any spore enlargement due to spore hydration would not have favoured the recombinant isolate. These results were however based on spores produced from only two isolation-bench pots; the humidity within these pots may have differed during the development of the pathogen and this could have affected spore size.

In the second experiment, 9 pots were used and watered regularly throughout the pathogen's development to try and maintain similar humidity levels between the pots. The spores were hydrated on 1% agar in a further attempt to ensure uniformity before measuring.

The spore volumes obtained in the second experiment were noticeably larger than in the first - probably due to the hydration. However, the mean spore size of MB-5 was still significantly larger than either of the other two races.

Day and Jones (1968) have shown that diploids of Ustilago violacea can be detected by the size of the conidia, but MacDonald et al. (1963) pointed out the need to assess spore size in each combination of strains in order to apply the size criterion to Penicillium crysogenum. Spore size differences were not however noted in Ustilago maydis (Holliday, 1961), although larger amounts of DNA were recorded from the diploid spores.

The results obtained from the first experiment show that the unhydrated spores of isolate MB-5 were almost twice the size of those of race 41 E 136, which suggests the possibility of isolate MB-5 being a diploid. However, from the second experiment, the difference in spore size was very much less. The experimental design could have contributed to this, as no allowance was made for the water absorbing properties of the spores. Ideally, spore measurements should have been made before and after hydration.

In view of this possible occurrence of a diploid stage it is appropriate to consider other evidence for this and its implication for the operation of the parasexual cycle. The parasexual cycle (Flor, 1964) has recently been discussed as a possible method by which recombination may occur asexually in the plant pathogenic fungi (Tinline and MacNeil, 1969), but usually this method has received less credence in relation to the rust fungi than other methods, mainly due to the lack of an observed diploid phase (Bridgmon, 1959; Little and Manners, 1969b). Diploid colonies have however been observed in axenic culture of P. graminis by Williams and Hartley (1971).

Ellingboe (1961) suggested that the diploid stage must exist even if it is only of a short duration. It would seem likely that this stage would occur within the host plant and would seldom produce diploid spores. This could account for the fact that a diploid phase has not been observed. The number of nuclei per spore, and the size of spore nuclei may be useful criteria for estimating ploidy, although Clutterbuck and Roper (1966) found that while the geometric means of the areas of diploid and haploid nuclei in Aspergillus nidulans differed in mean area by a factor of 1.75, an overlap in size distribution precluded differentiating them in heterokaryons. Cytological studies of the MB-5 isolate showed no differences in the size or number of nuclei when compared with the 'parental' races. Spore size in this case is evidently independent of nuclear size and number, and may not be related to ploidy level.

The generally larger spore size of MB-5 could be indicative of a more agressive isolate with larger food reserves, which could provide greater reproductive opportunities, if the survival period between germination and penetration is increased as a result of increased food reserves within the spores. If this were the case, the penetration frequency of isolate MB-5 should be higher than that of the parental races.

The number of spores which are successfully established as single spore isolates is extremely small when compared with the total number of spores produced by a single leaf. In this work, 20 pots of 8 seedlings were used for the initial mixed inoculations, so the importance of screening the population becomes greater.

Two facts emerge using the methods outlined in section I.2xa: firstly the approximate composition of the spore population in terms of the two parental races; and secondly whether any recombinant isolates of increased virulence are present.

The first inoculation gives a good indication of the race composition of the spore mixture. From Table I.4 it can be seen that spores from M. Beacon gave rise to a high percentage of susceptible infections on Hybrid 46, whereas only a few Heine 110 seedlings were susceptible to the spore mixture. The reverse is true for the spores off M. Envoy, and both results are expected, as Maris Beacon is both susceptible to race 104 E 137 and resistant to 41 E 136; the opposite is true of M. Envoy and Heine 110. Both Michigan Amber and Maris Nimrod are susceptible to both races, and equally good infections on both Hybrid 46 and Heine 110 would be expected. However, the spores from Michigan Amber did not illustrate this, but gave a very low level of susceptible Heine 110 seedlings. This suggests that the race mixture was predominantly race 104 E 137, despite both races being applied in approximately equal amounts. This would therefore reflect on the germination of the two parental races. From Table I.7 it can be seen that all single spore isolates from Michigan Amber were races 104 E 137, which would again support the unequal distribution of spores in this collection. The possible presence of recombinant isolates is determined in the second inoculations as the spore collections from the first inoculations should have reduced the spore composition to predominantly one race, with only a few 'contaminant' spores of the other race being present in each collection, as illustrated by the spores originally from Maris Nimrod (Table I.5).

From Table I.5 however, the three infections marked with an asterisk deviated from this. These were suspected of containing some recombinant isolates. When tested on the F_3 material, the spores from Michigan Amber

did not give rise to any isolates showing recombined virulence: from the spores off Maris Beacon, 3 F_3 seedlings gave a low level of sporulation, and a single infection point was observed from the spores off Maris Envoy, suggesting the recombined virulence. These isolates were not tested on the differential sets, but their ability to infect the F_3 would suggest that they were the new race 105 E 137.

The high number of suspected recombinant isolates, especially in the isolates established early in the work, was probably due to the isolation chambers used before the isolation bench was built.

The isolation of a contaminant spore of race 60 on Heines Kolben illustrated one of the hygiene problems when handling airborne pathogens. No work had been carried out using this race during the 4 months preceding its isolation, so contamination from another glasshouse compartment can be excluded. This leaves 3 alternatives: either the spore arose by mutation, or was produced from the mixed inoculations in use in this work, or else, it had been brought into the glasshouse from outside, probably on clothing, possibly following the inoculation of field plots.

Several of the single spore isolate cultures showed differences in their time from inoculation to the first signs of infection and to sporulation. From similar work using 9 American races of P. striiformis, Beaver (1969) was able to create 3 race groups, based on the time taken from inoculation to sporulation, into which his 9 test races could be grouped. Such groupings are not possible from the work described here, however, as the experiments were carried out in the glasshouse and not in controlled environment cabinets as had originally been planned. Thus, comparisons of isolates were possible within the experiments, but not between experiments. For this reason, only isolates which differ significantly from the parental races may be considered.

Only two races, race 41 E 136 and 104 E 137, and isolates obtained from these races were considered. Of the three recombinant isolates (race 105 E 137) tested, isolate MB-5 showed a slower incubation period, MB-6 did not differ significantly at either stage of its development, and isolate MN-7 had a shorter lag time, between flecking and sporulation. Similar observations were made from the parental races 41 E 136 and 104 E 137, the single spore isolates from these showing a variety of development times. This would support Beaver's (1969) observation that no correlation could be made between incubation or latent periods for an isolate of this rust, and its characterization as a race. Some isolates were significantly slower than the 'parental' race in one experiment and not in later experiments when the isolate was retested. This would support Zadoks (1961) observation that the time to sporulation varied randomly in experiments with P. striiformis. Van de Plank (1963) discussed ways in which many minor environmental influences before and during development can affect the rate of development of each individual isolate, and he suggested that the rate of development can vary even when the interaction of host, pathogen and environment is constant.

One important possibility affecting this variation is the effective dose of inoculum, as although approximately equal volumes of spores were used, the level of germination between isolates is seldom constant, and this could easily vary randomly between and within experiments.

Beaver (1969) found that a temperature change from 18°C to 2/18°C had little influence on the time lag between symptom development and sporulation, but suggested that it was the main factor influencing the latent period by the changing of the length of the incubation period.

From this work the increase in temperature (Table I.10) resulted in the shorter time for both the development of symptoms and the start of sporulation, and a less marked reduction in the lag time. This would support the evidence of Beaver (1969) in which the time up to the development of infection symptoms was the critical factor which in turn affected the time to sporulation. Further supporting evidence from the histological studies is discussed later.

An influence of varietal resistance on speed of development was noted, and the incubations and latent periods recorded on the different varieties under uniform conditions was found to be indicative of the pathogen's suitability or compatibility to the host on which it was growing, as illustrated with isolates 71/2 and 72/40.

Johnson and Taylor (1972) suggested that an isolate (71/2) of race 104 E 137, collected off the variety Joss Cambier, was better adapted to this variety than to Hybrid 46 and that the older isolate (69/10) of race 104 E 137 was better adapted to Hybrid 46 than to Joss Cambier.

Isolate 71/2 and another isolate from Joss Cambier, 72/40 (race 41 E 136) were used in one of the experiments, and the results are summarised in Table I.13. The data agree with the observation of Johnson and Taylor (1972) that 71/2 is better adapted to Joss Cambier, and also shows that 72/40 was similarly better adapted to Joss Cambier than to the variety Heine 110.

Beaver (1969) also observed the influence of reaction type on latent period, but did not consider the incubation period. The time to sporulation is of great importance, as a pathogen with a short cycle time will be able to produce a greater number of generations within a given time. If as in the case with isolate 71/2 on Joss Cambier a greater number of spores are also produced, (Johnson and Taylor, 1972) the pathogen's epidemiological advantage would be considerable.

Table I.13. Incubation, latent and lag periods obtained using isolates 71/2 and 72/40 of P. striiformis, on three of the test varieties

Varieties	Days					
	Incubation period		Latent period		Lag period	
	71/2	72/40	71/2	72/40	71/2	72/40
Hybrid 46	5.5	5.7	10.6	*	5.0	*
Heine 110	6.0	6.4	*	11.1	*	4.7
Joss Cambier	5.8	5.9	10.0	10.3	4.2	4.5

Underscoring - susceptible reactions
* - missing plot values

Fuchs (1966) observed that 'race' isolates from different origins may differ in incubation period and suggested that such differences may be confirmed as true race differences with the addition of suitable supplementary varieties. It is interesting, however, that similar variation in incubation time is observed within isolates taken from a pure race culture, obtained from a single spore.

Any evidence of consistence in differences between isolates within races for cycle time could therefore consititute an important aspect of variation between isolates varying independently of the genes for virulence.

II. HISTOLOGY

Introduction

The two types of resistant reaction (0 and 00) recorded earlier may have a significant role in the origins of new races, as the micro-environment in which the pathogen develops could afford a considerable selection pressure on the race or races present. These experiments are to try and establish the types of host environment available to the pathogen, and to suggest which of the different environments would be most suitable for the initiation and development of new races.

Studies of the rusts *in vivo* have been carried out since the end of the 19th Century, in an attempt to establish and explain the different responses induced in the host by various races of the pathogen. As relatively little work has been published concerning *Puccinia striiformis*, other rust species will serve as examples to illustrate some generally accepted theories and ideas on the resistance and susceptibility of host varieties.

The main stages in the asexual cycle of the rusts may be outlined as follows:

i) Pre-penetration, i.e. spore germination and germ tube growth.

ii) Penetration.

iii) Post-penetration, which may be subdivided into:

mycelial development and

spore formation and liberation.

Each stage has at some time been considered critical in terms of resistance.

II1. Literature.

1a. Pre-penetration phase

Spore germination is reported on artificial media, (Rodenhiser and Hurd-Karrar, 1947; Manners, 1950; Tollenaar and Houston, 1965), as well as on resistant and susceptible hosts (Wellensiek, 1927; Wilcoxson, 1957; Flentje, 1959; Rothmon, 1960). White (1964) proposed two types of inhibition effective against germination: self inhibition, discussed in Chapter 3, and axenic inhibition. He showed that axenic inhibition could arise from the host, and that guttation drops and leaf extracts influenced germination, but he found that they could not be correlated with host resistance.

1b. Penetration

It has been shown that the germ tubes enter the host primarily through the stomata (Flentje, 1959; Wilcoxson, 1957). Flentje reported that penetration reactions were probably caused by some stimulus associated with the host surface, which triggered appressorial development, but Weber (1922) was unable to show any kind of attraction of germ tubes towards stomata during initial stages of growth. Allen (1956), detected the presence of a volatile substance produced by germinating spores of P. graminis which inhibited germ tube elongation, and induced the formation of appressoria and infection structures in other uredospores floating on liquid. He did not however relate this to the infection of host tissues.

The possibility of diurnal stomatal movements influencing penetration was considered by White (1964), but he concluded that penetration was conditioned by qualities of the host other than stomatal behaviour. Brown and Shipton (1964) also found no effect of stomatal behaviour on

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penetration, as different races did not follow similar infection patterns, which would be expected if stomatal movements influenced penetration.

Wellensiek (1927) and Rothmon (1960) reported that in P. sorghi and P. coronata respectively, the initial stages of infection were similar in susceptible and resistant reactions, but Rothmon suggested that the substomatal vesicle was more characteristic of an incompatible reaction, and served as a resting organ for the pathogen during the two days following penetration in which the fungus does not grow. Brown and Shipton (1964) found no correlation between penetration and infection type or the number of pustules in P. graminis tritici.

Romig (1964) has illustrated a high degree of stomatal exclusion of P. recondita on the peduncles and leaf sheaths of some oat varieties, although the laminae were readily penetrated, illustrating an interesting protection of the reproductive parts. He suggested that this could be due to the thicker guard cell walls affording resistance to penetration. Ogle and Brown (1971) observed no penetration of P. graminis on non-host species, but Hilu (1965) observed initial infections on barley, oat, soybean and wheat, when inoculated with P. sorghi.

1c. Post-penetration phase

Controversy about the relationship between resistance and cell collapse has continued since 1902.

The earliest theories to explain the mechanisms affecting the exhibited degrees of resistance to the rusts were that they were mechanical. It was suggested that resistance was caused by morphological characters such as stomatal size, waxy covering and thickness of the cuticle (Cobb, 1890; Hitchcock and Carleton, 1893). Detailed histological studies by Ward (1902), on Bromus species infected with P. dispersa,

however, invalidated that theory and indicated that resistance was dependent on the interaction of the protoplasm of host and fungus, which results in necrosis. He accounted for the differences in adaptibility by the difference in the rate of starvation of the mycelium, which prevents the pathogen from spreading through the host. Gibson (1904) and Bolley (1908) attributed the death of the hyphae to toxins emitted solely from the host, whereas Marryat (1907) suggested that mutually destructive toxins from the host and the pathogen caused the reaction.

Stakman (1914) observed this association between colonisation of leaf tissue and necrosis in P. graminis. He attributed the death of the host cells directly to the presence of the hyphae and in 1915 he proposed the term 'hypersensitivity' to describe the reaction. He suggested that immunity was due to a physiological incompatibility which resulted in the abnormally rapid death of the host cells when attacked by the rust hyphae.

Newton (1922) suggested that the susceptible host adjusts itself to the presence of the parasite, whereas the resistant host remains intolerant of it. Wellensiek (1927) described the difference between resistance and susceptibility as the difference in the amount of necrosis prior to spore formation in the host.

Sharp and Emge (1958) reported a 'tissue transplant' technique by which hypersensitive tissue caused by P. graminis on a resistant variety, could be transferred to a susceptible host. They obtained spores from the culture and used them to identify the races causing resistance. This illustrated that the pathogen was not killed, but that its growth was only arrested by the hypersensitive reaction. This was also illustrated in P. coronata (Zimmer and Schafer, 1961). Littlefield and Aronson (1969),

however, were unable to obtain spores from Melamspora lini using the technique, possibly suggesting that in this case the mycelium had been killed.

Forsyth and Samborski (1958) found that P. graminis was still viable following the resistant reaction on Khapli, and they were able to break the resistance by detaching the leaves and searing the base of the leaf with a hot spatula. This resulted in an increase in nitrogen and soluble carbohydrates, and induced susceptibility. They suggested that the change in resistance could have been due to the removal of an inhibitor or the increase in the available essential substrates and that substrate availability was of major importance in rust development.

Work on P. coronata (Rothmon, 1960) and P. sorghi (Hilu, 1965) and P. graminis (Brown et al., 1966; Leath and Rowell, 1966; Ogle and Brown, 1971) led the authors to believe that necrosis and cell collapse are consequences rather than causes of resistance, and Leath and Rowell point out that the fungal growth ceases before the possible nutrients are exhausted. White (1964) suggested that in P. graminis the amount of cell necrosis could be considered as an indication of the intensity of the reaction, but Ogle and Brown (1971) found that this was not so, and that the largest areas of necrotic tissue were associated with the intermediate reaction.

II2. Experimental Methods and Materials

Seedlings were grown and inoculated as described above (Ch. I2.iiid). The varieties and races used are given in Table II.1 together with their seedling reactions to the races concerned.

Table II.1. Host Varieties and races of P. striiformis used, with expected seedling reactions to the races with which they were inoculated

Variety	Race 41 E 136	Race 104 E 137
Maris Beacon		4
Maris Nimrod		4
Maris Envoy		00
Heines Kolben		0
Hybrid 46	0	4
Heine 110	4	00

The inoculated seedling leaves were harvested 24 and 48 hours, 5, 10 and 15 days after inoculation and prepared for observation.

Anderson and Rowell (1962) reported a spray staining method to observe germinated spores on a leaf surface. The stain contained acid fuchsin and cotton blue, and was reported to stain blue those hyphae which had achieved penetration and red those which had not, thus permitting them to be distinguished. Differential staining of the germ tubes did not occur when I used the method and the preparations deteriorated rapidly. I therefore used the whole leaf clearing technique of Shipton and Brown (1962).

Lengths of infected leaves were boiled in alcoholic lactophenol cotton blue (Shipton and Brown, 1962), for about 2 minutes and left to cool. They were stored for at least 48 hours at room temperature before examining, to ensure that the stain had penetrated the mycelium within the leaf tissue. The leaves were placed in chloral hydrate solution for 1-24 hours immediately before examination, to remove excess stain from the leaf tissue, though this step proved unnecessary and was sometimes omitted. The leaves were mounted in 50% glycerine and the adaxial surface was studied at a magnification of 400x.

The numbers of germinated and ungerminated spores and observed number of penetration points contained in a measured leaf area were recorded for all the varieties listed in Table II.2 at 24 and 48 hours after inoculation, during which the plants were maintained at 7.5°C. Although some spores had probably been washed off the leaves during the leaf clearing technique, it was assumed that the fraction lost in this way would be similar in all treatments.

After 5, 10 and 15 days, the presence of penetration points, internal mycelium, necrosis and young pustules was recorded by taking observations at 0.5 mm intervals over the leaf surface, scoring the character as present or absent and plotting the result on to a scaled plan of the leaf segment.

II3. Results

i) Pre-penetration

Germination of race 104 E 137 on Maris Beacon, Maris Nimrod, Maris Envoy and Heine Kolben was about 25%, but was significantly lower on Maris Envoy (12.1%). This could suggest that some inhibitor is present in Maris Envoy. There were, however, similar numbers of infection points on Maris Envoy, Maris Nimrod and Maris Beacon. In the case of Maris Envoy, the penetration points were associated with a high level of necrosis, not encountered with the other two varieties. Heines Kolben showed restriction in the establishment of penetration points, and although considerable necrosis was observed, it was very much less than in Maris Envoy. Maris Beacon and Maris Nimrod, both of which are susceptible to race 104 E 137 showed very little necrosis (Table II.2).

Table II.2. Germination, infection and associated necrosis in four varieties inoculated with race 104 E 137

Variety	Percentage Germination	Percentage Infection Points	Percentage Necrosis
Maris Envoy	12.1	12.5	71.4
Heines Kolben	27.4	3.2	25.0
Maris Beacon	24.9	10.2	5.0
Maris Nimrod	24.0	17.8	4.7
5% L.S.D.	12.55	15.04	40.74

Percentage germination of race 41 E 136 on Heine 110 and Hybrid 46 was 24.9 and 27.3% and of race 104 E 137 was 8.1 and 10.4%. The lower infection with race 104 E 137 however, facilitated studies, as individual colonies could be observed. Comparisons made between 24 and 48 hours cold treatment showed a consistently higher number of penetrations after 48 hours than 24 hours. There were significantly more penetration points ($p = 0.05$) on Hybrid 46 than on Heine 110, Table II.3. The number of penetration points showed no correlation with spore density or germination.

Table II.3. Percentage infection points obtained after 24 and 48 hours cold treatment during inoculation

Variety	Race	24 hours	48 hours
Hybrid 46	41 E 136	20.6	44.4
Heine 110	41 E 136	8.3	24.0
Hybrid 46	104 E 137	14.3	28.6
Heine 110	104 E 137	11.1	20.0

L.S.D. between treatments = 9.79 at 5%

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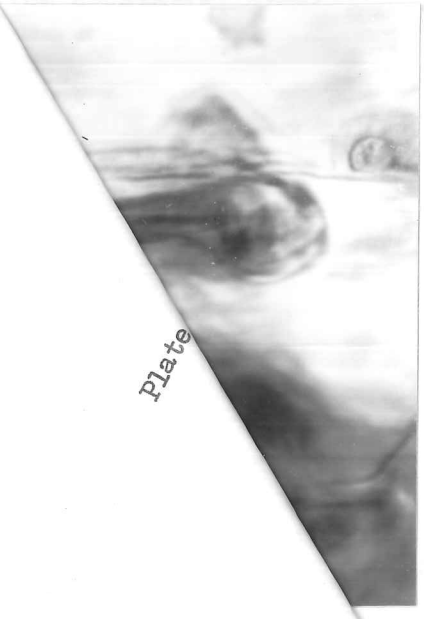

Plate

ii) Penetration

Large substomatal vesicles associated with penetrations have been observed in all the varieties studied and the fungus made little growth during the following 2 or 3 days. They did not appear to be any more common in resistant hosts and could be a resting organ as described by Rothmon (1960) in P. coronata. In Maris Nimrod, some penetration points were observed with no vesicles, and in these cases, the two infection hyphae produced were longer than in those with vesicles, which suggested the absence of a rest period following penetration, but little colonisation was observed before 10 days as in the other cases.

Vesicles were generally located at one end of the stoma and produced two infection hyphae: of these, the one growing towards the rib of the leaf developed more rapidly than the one growing away from the rib (Plates II.1a and II.1b). The orientation of the vesicle and preferential growth of the hyphal initial towards the leaf rib could be the result

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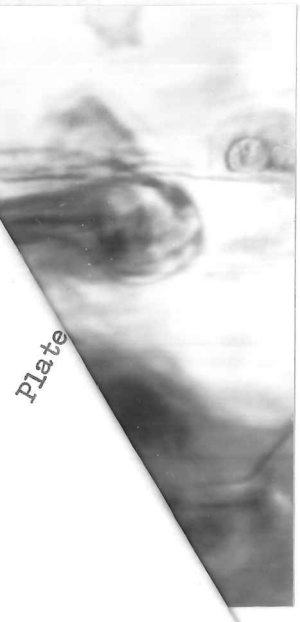

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Germ tubes were usually observed running across the leaf axis, rather than along it. When a germ tube encountered a stoma it grew along the longitudinal axis of the stoma. It did not always penetrate the first stoma reached, but in some cases continued to grow until reaching another. This would suggest that the condition of the stoma has some influence on the control of penetration, and that stomatal state could be critical to the establishment of infection. Unlike P. graminis and P. hordei, no noticeable appressorium is developed in P. striiformis, and penetration is achieved directly by the germ tube tip. It is possible that the fungus is therefore less able to force open the stoma, and that it relies on locating an open stoma for penetration. Germ tube fusions were observed on the leaf surface.

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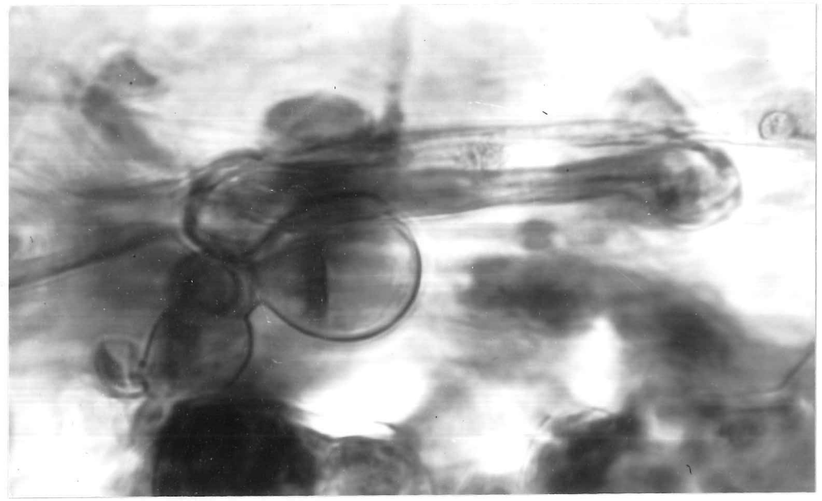
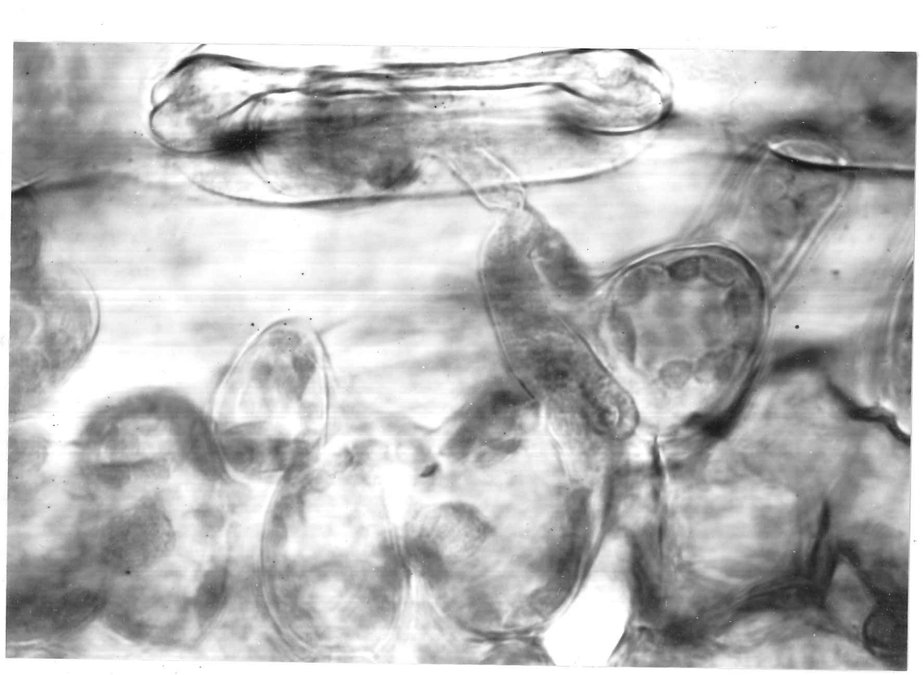
Plate II.1a. Penetration point giving rise to large substomatal vesicle. Hyphal initials are also visible (x 1000).

Plate II.1b. Penetration point. Hyphal development towards rib. (x 1000).

a



b



of nutrient diffusion from the phloem or of the greater cell density affording closer cell contact and more sites for haustorial connections.

Necrosis was evident around the penetration points of Maris Envoy, HeinesKolben and Heine 110 inoculated with race 104 E 137 (Plate II.2a), and in Hybrid 46 inoculated with race 41 E 136. Necrosis was more intense in Heine 110 and Maris Envoy, affecting nearly all the immediately adjacent cells, than it was in HeinesKolben and Hybrid 46 where less than half of the adjacent cells were necrotic, and where necrosis was not associated with all the penetrations. Maris Beacon, Maris Nimrod and Hybrid 46 showed very little necrosis when inoculated with race 104 E 137 and Heine 110 had little necrosis when inoculated with race 41 E 136.

iii) Post-penetration phase

a) Development in susceptible hosts. Following penetration, there was little growth of the pathogen during the next few days, and the extent of growth by five days after inoculation was minimal. Only in Heine 110 had mycelial growth extended more than 0.5 mm (Fig. II.1).

Very little necrosis was observed in the susceptible hosts throughout the development of the pathogen, until pustule formation was fairly extensive. At this stage, there was some cell collapse between pustules which may have been caused by the pressure created within the leaf tissue by the enlarging spore masses. The mycelium did not appear to be affected.

The most rapid growth of the pathogen took place between five and ten days after inoculation. During this time the fungal hyphae developed an extensive mycelium among the host cells (Plates II.3a and II.3b). The pathogen was totally confined to the mesophyll cells and

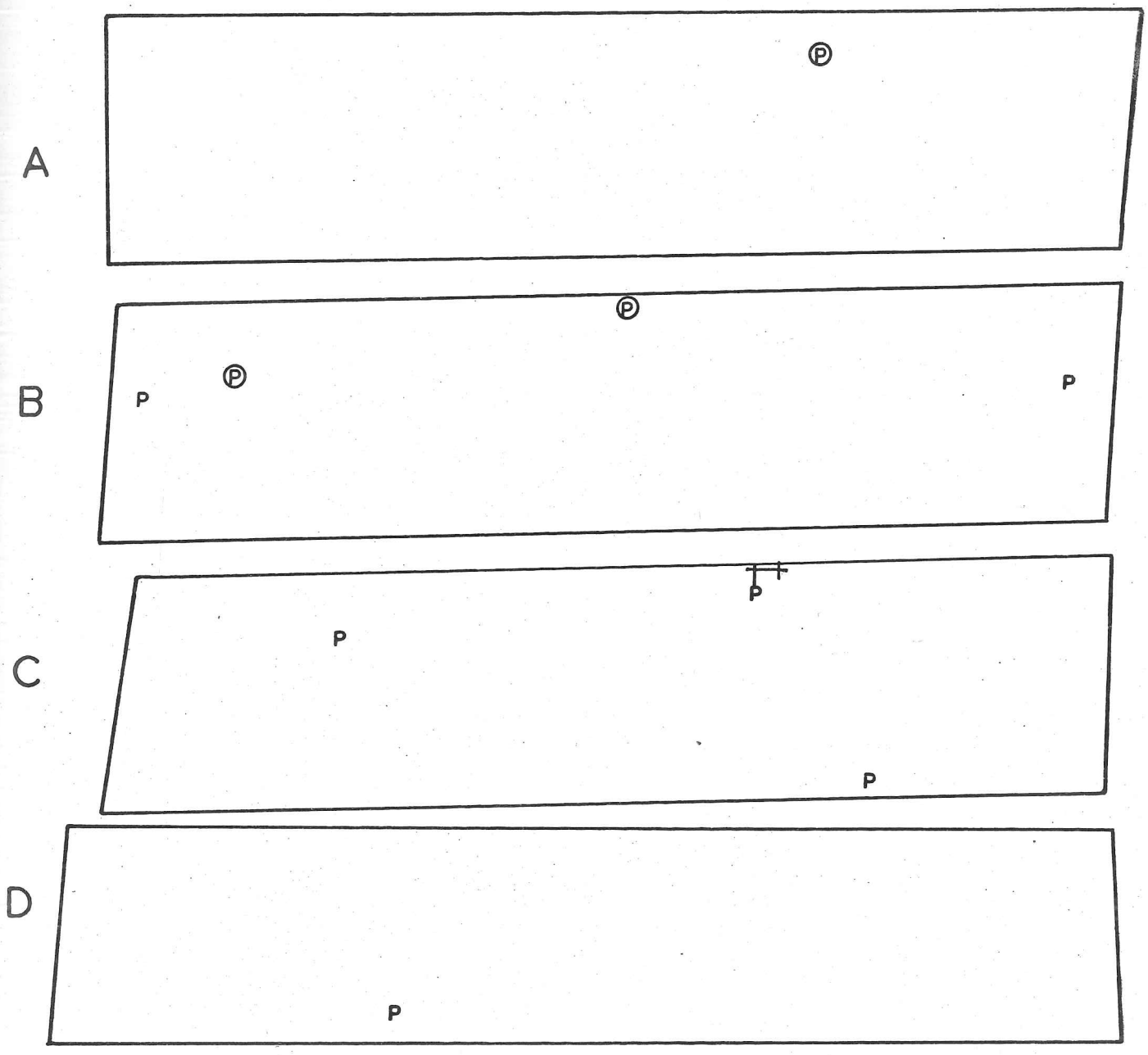


FIG II.1 RELATIVE SPREAD OF PUCCINIA STRIIFORMIS IN HOST. 5 DAYS AFTER INOCULATION. A. HEINE 110 WITH RACE 104 E 137 B. HYBRID 46 WITH RACE 41 E 136 C. HEINE 110 WITH RACE 41 E 136 D. HYBRID 46 WITH RACE 104 E 137 P. PENETRATION POINT WITHOUT & Ⓟ WITH NECROSIS. +. MYCELIUM PRESENT. SCALE ⊢⊣ 1 MM.

Plate II.2a. Penetration point. Early stages in resistance following penetration of Heines Kolben with race 104 E 137 (x 1000).

a

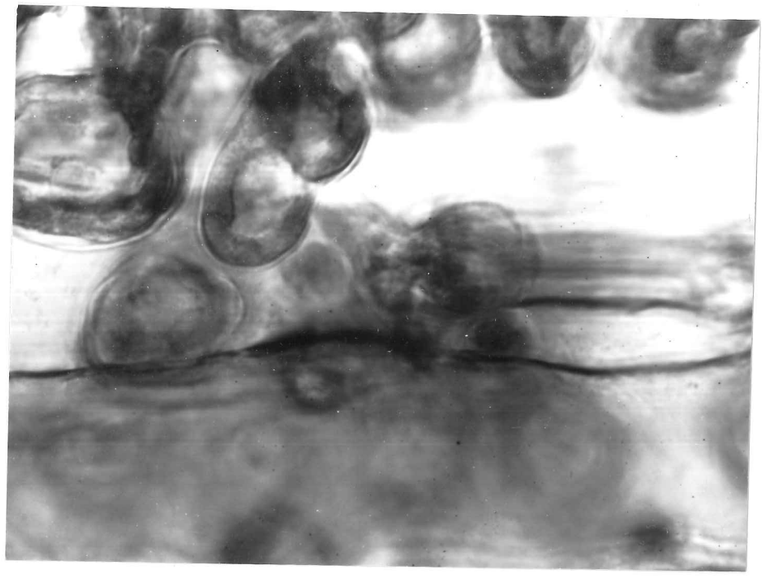


Plate II.2b. Runner hyphae(a) with lateral infection hypha(b) and haustorial connection in epidermis(c) (x 1000).

b

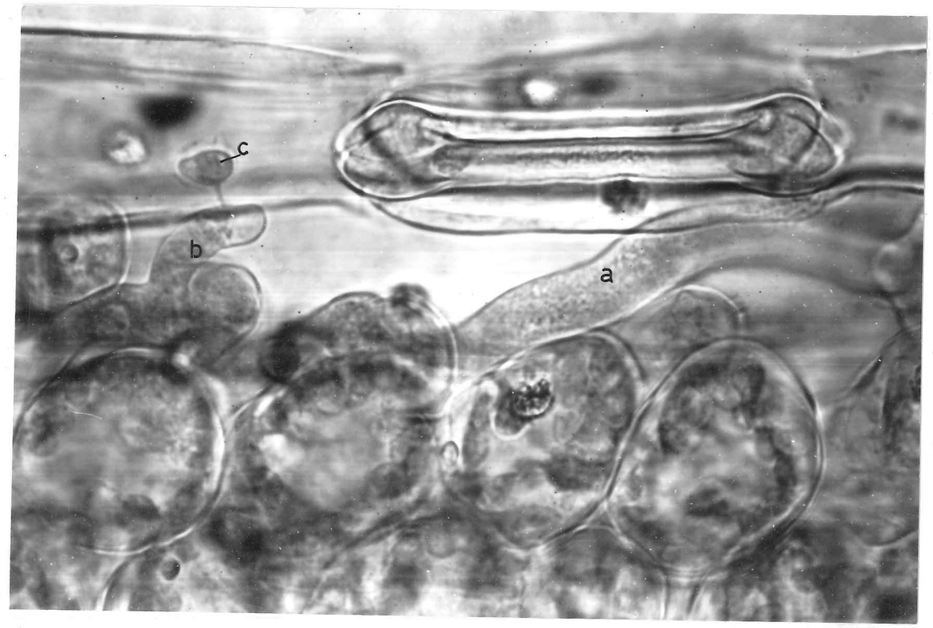
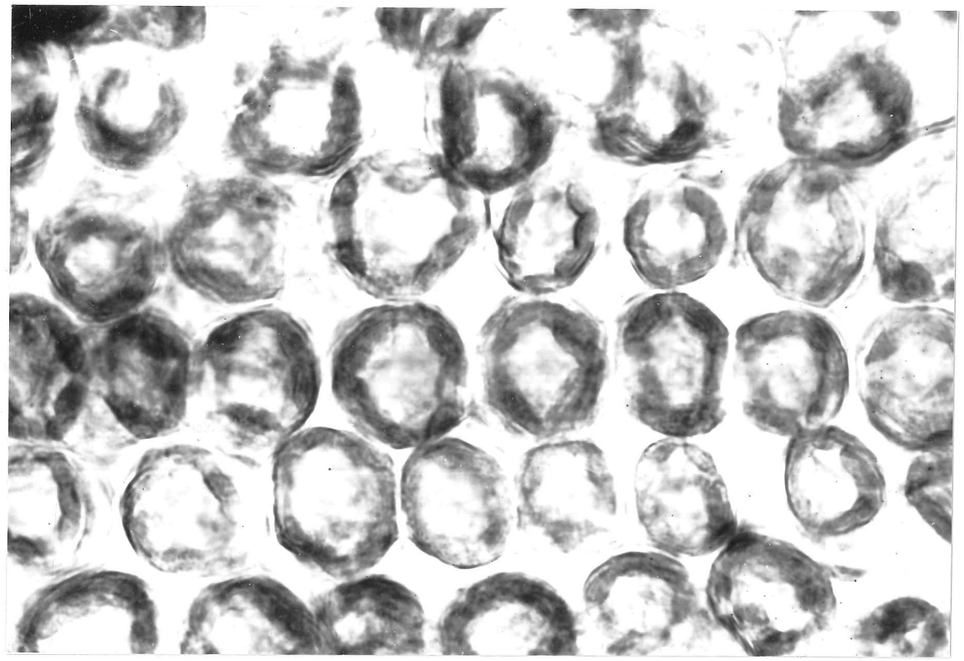


Plate II.3a. Uninfected mesophyll tissues (x 1000).

Plate II.3b. Fungal mycelium completely filling the intercellular spaces between mesophyll cells (x 1000).

a



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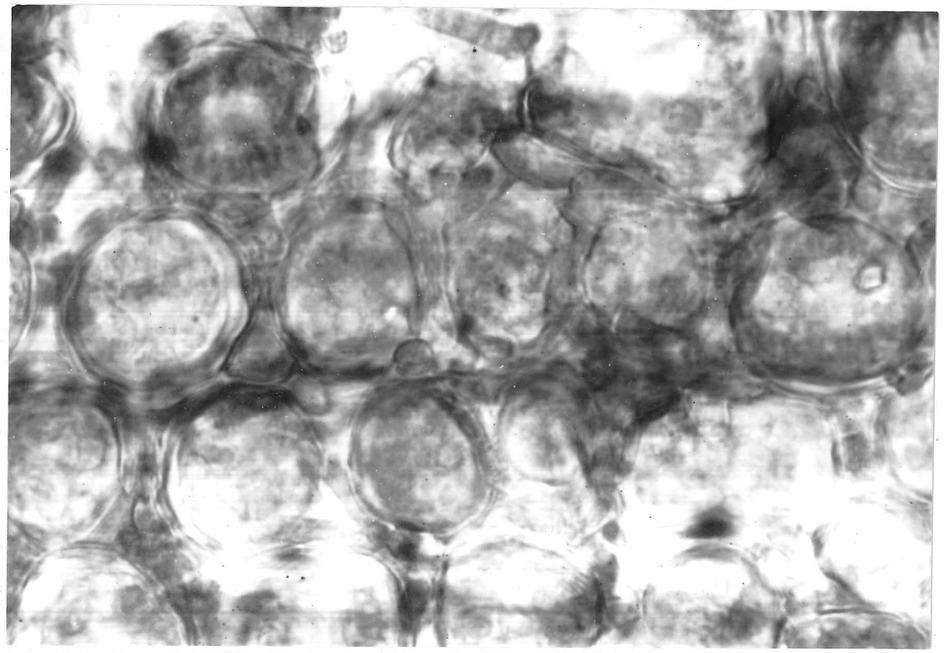


there was no evidence of invasion of the vascular bundles or their sheaths.

Three types of hyphae have been observed:

Runner hyphae,

Infection hyphae,

Spore initiating hyphae.

Runner hyphae (Plate II.2b) are the largest hyphae (11.7 ± 1.1 μm in diameter) and have few cross walls. They tend to precede the infection hyphae and run along the axis of the leaf close to the rib, entering previously uninfected tissue. They show little branching but give rise to lateral hyphae which are the infection hyphae.

Infection hyphae are considerably smaller than the runner hyphae (8.2 ± 0.7 μm in diameter). They branch frequently, have many cross walls and can surround mesophyll cells, (Plate II.4a). These hyphae develop haustorial connections with the adjacent cells and haustoria have been observed in epidermal cells as well as mesophyll cells, (Plates II.2b and II.4b and c). Infection hyphae form the greater part of the mycelium and give rise to the spore initiating hyphae.

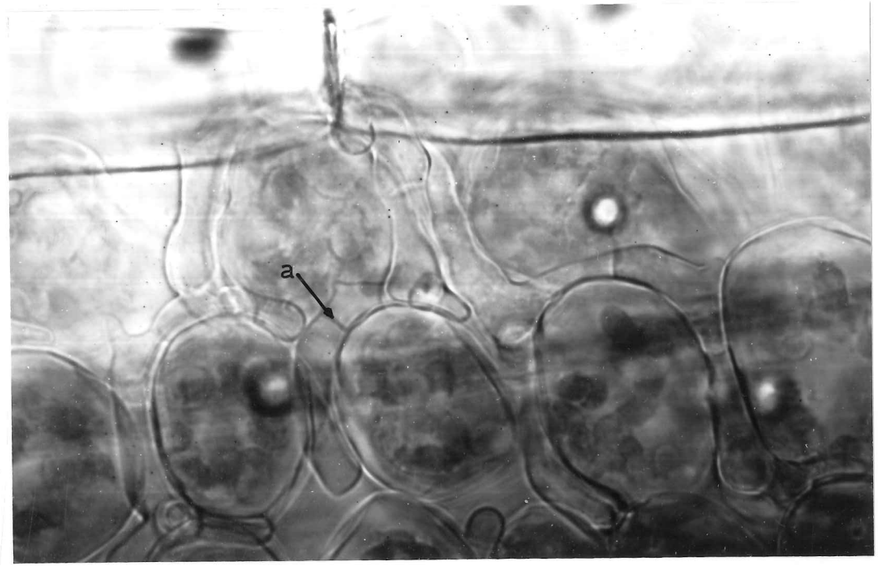
The spore initiating hyphae are differentiated from the infection hyphae by being finer (4.9 ± 1.1 μm in diameter) and possessing a greater number of cross walls (Plate II.5a). These hyphae form a dense hyphal network between the mesophyll cells and the epidermis, and fusions have been observed between hyphal tips (Plate II.5b). The tip of these hyphae start to enlarge, and the cell wall becomes thicker, until a young spore is recognisable. These spores develop in masses, forming pustules and continue to grow until mature spores are visible under the epidermis (Plate II.6a).

Plate II.4a. Mycelial cross walls (a) and branching round mesophyll cells (x 1000).

Plate II.4b. Haustorial initials into mesophyll cell (x 1000).

Plate II.4c. Haustorial connection with mesophyll cell (x 1000).

a



b

c

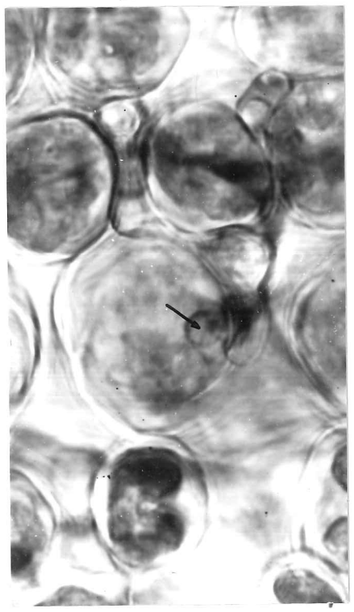
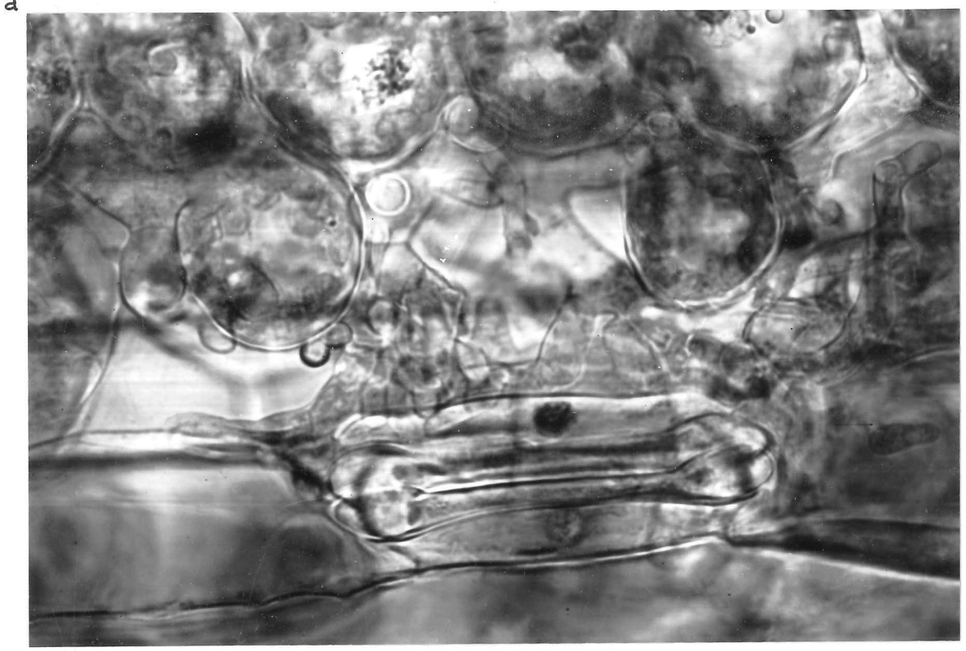


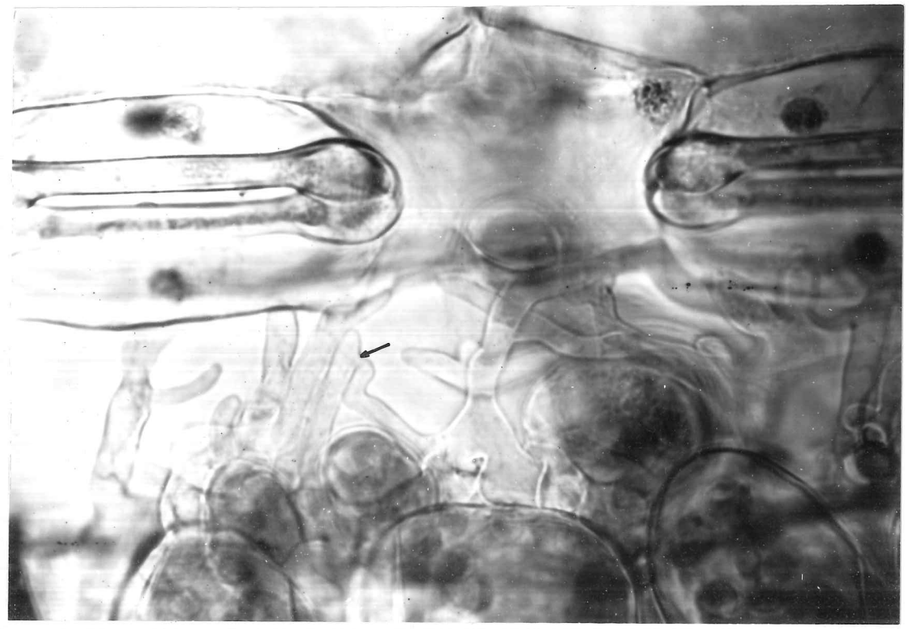
Plate II.5a. Densely packed mycelium prior to spore initiation and formation (x 1000).

Plate II.5b. Dense hyphal mat with fusion between two hyphal tips (x 1000).

a



b



The epidermis ruptures, probably as the result of the pressure created by the pustules, and releases the spores. There is no visible evidence that the spores produce a substance to dissolve or weaken the epidermal cells, and the edges of the ruptured epidermis are uneven and have a torn appearance.

Spore and pustule development started sometime between 8 and 10 days after inoculation in Heine 110 infected with race 41 E 136, and in Maris Nimrod infected with race 104 E 137, and between 10 and 15 days in Hybrid 46 and Maris Beacon infected with race 104 E 137. The rate of development of the pathogen could be indicative of the level of compatibility between the host and pathogen.

Mycelium was observed up to 10 mm in front of the sporulating tissue and the pustules in the front of the colony showed a banded pattern, possibly reflecting a diurnal cycle in the development of the pathogen.

b) Development in resistant hosts. Some level of necrosis is associated with the resistant reaction throughout its development. In the O type of resistance, many, but not all the penetration points exhibit necrosis, and where present, only a few cells are affected. In all cases however, necrosis develops as the mycelium starts to spread.

In the type OO reaction, necrosis is nearly always observed surrounding the penetration point. Necrosis occurs in cells in immediate contact with the mycelium, to a depth of 2 or 3 cells, (Plate II.6b) and this results in the complete limitation of mycelial growth, and effectively isolates the pathogen from healthy leaf tissue. Runner hyphae have not been observed to develop from mycelium contained in a OO reaction type, and the colonies show little development after 5 or 10 days (Fig. II.2 and II.3).

Plate II.6a. Pustules visible under the epidermis (x 200).

a

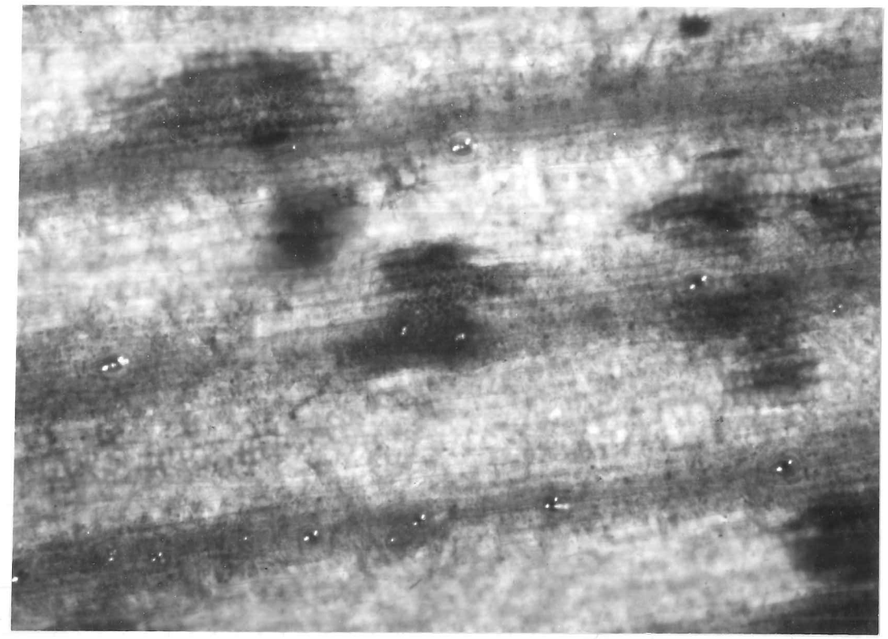
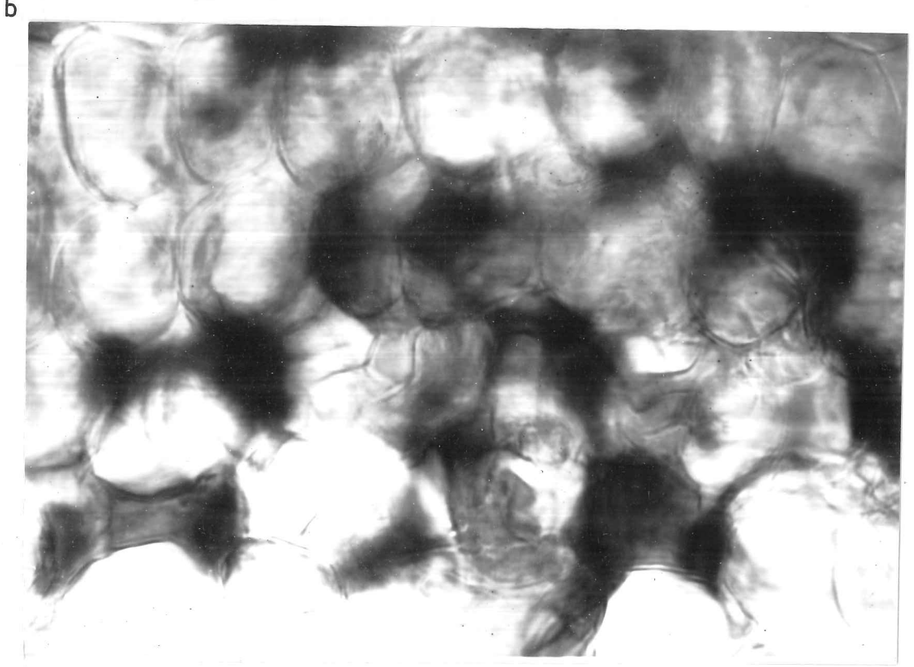


Plate II.6b. Necrosis in a 00 reaction type affecting high percentage of cells around mycelium (x 1000).

b



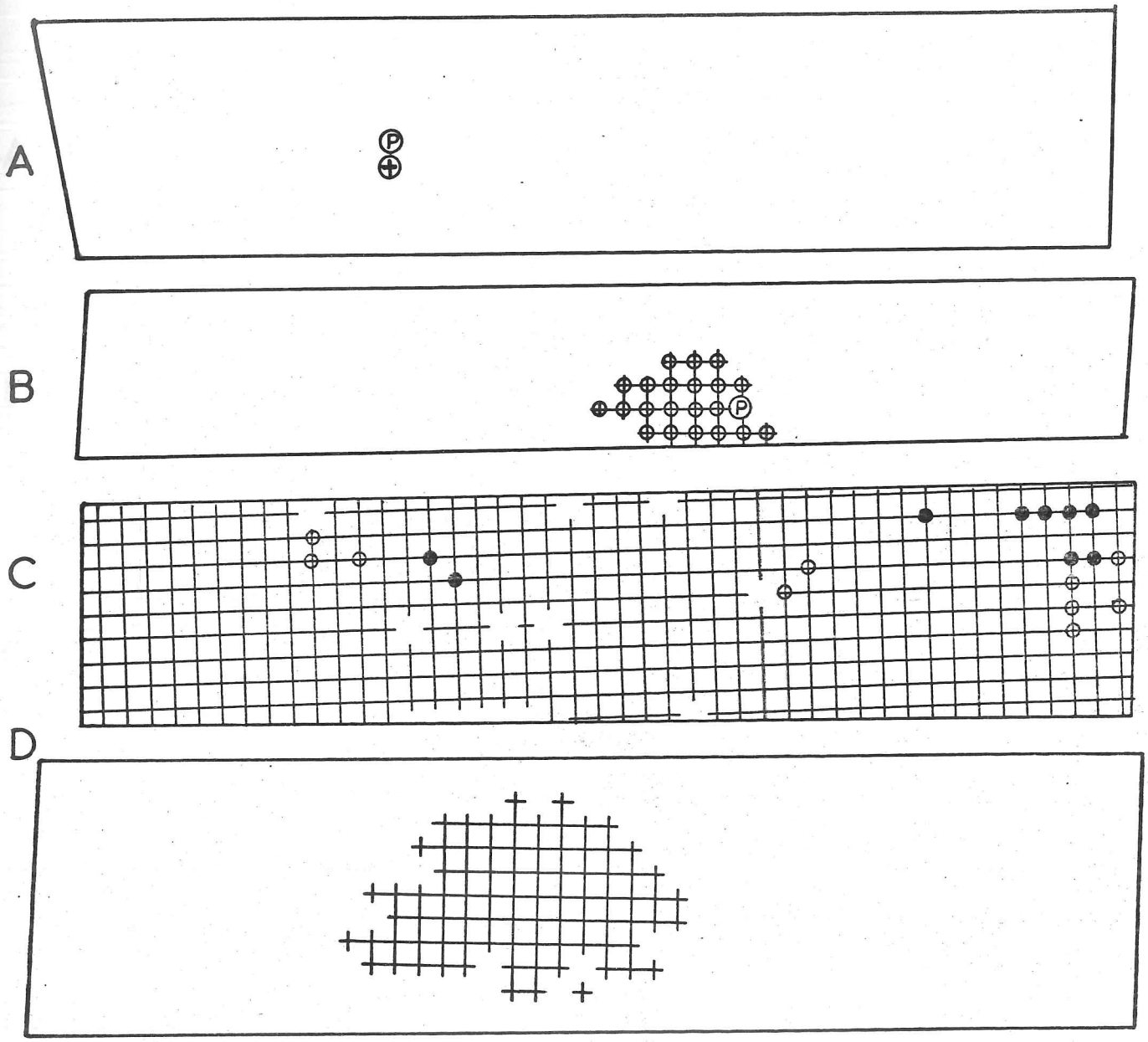


FIG. II. 2 RELATIVE SPREAD OF PUCCINIA STRIIFORMIS IN HOST, 10 DAYS AFTER INOCULATION. A. HEINE 110 WITH RACE 104-E-137 B. HYBRID 46 WITH RACE 41 E 136 C. HEINE 110 WITH RACE 41 E 136 D. HYBRID 46 WITH RACE 104 E 137. P. PENETRATION POINT WITHOUT & Ⓟ WITH NECROSIS. + MYCELIUM PRESENT WITHOUT & ⊕ WITH NECROSIS. ●. PUSTULES PRESENT. SCALE ⊢—⊣ 1 MM.

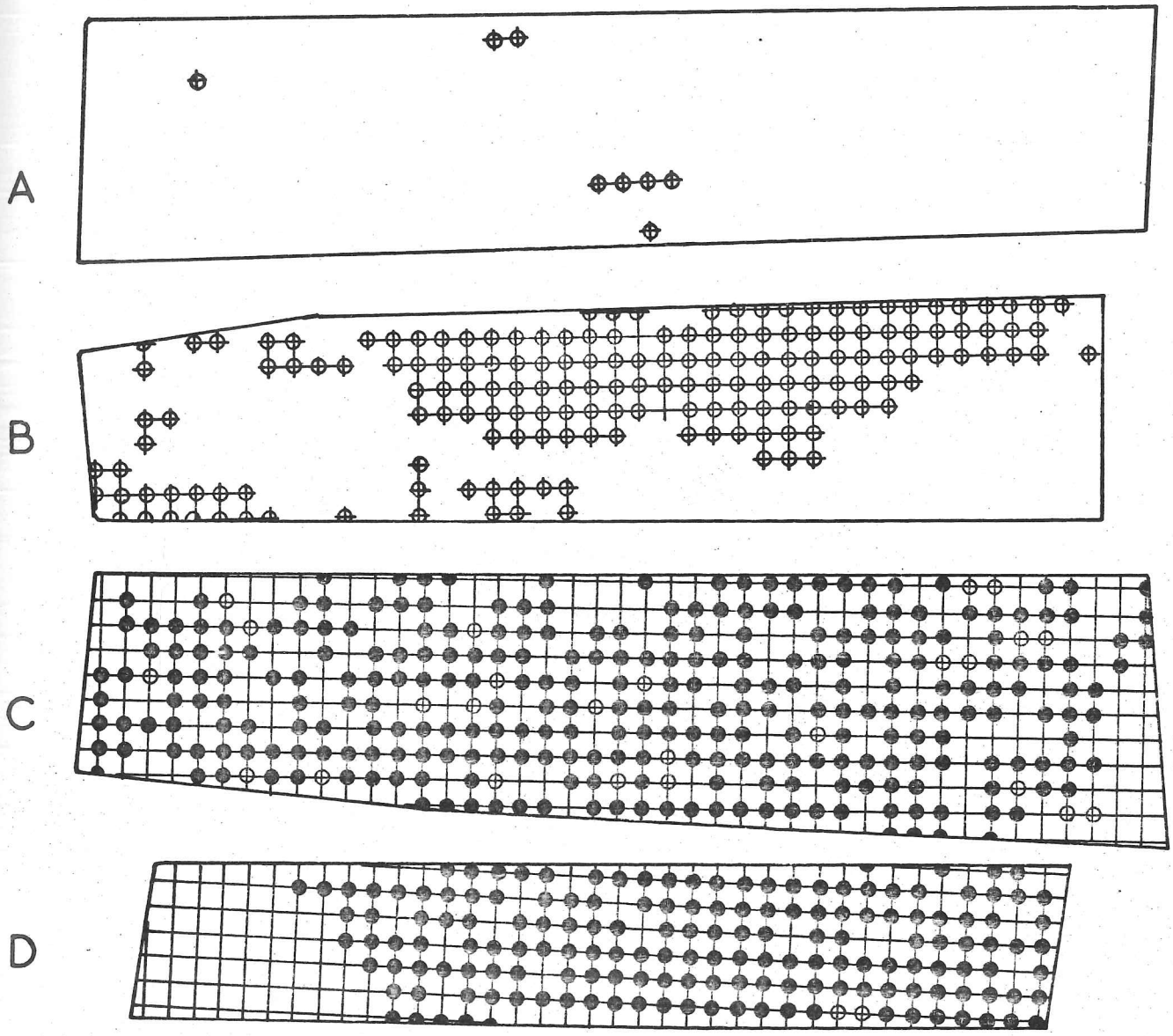


FIG. II. 3 RELATIVE SPREAD OF PUCCINIA STRIIFORMIS IN HOST. 15 DAYS AFTER INOCULATION. A. HEINE 110 WITH RACE 104 E 137 B. HYBRID 46 WITH 41 E 136 C. HEINE 110 WITH RACE 41 E 136 D. HYBRID 46 WITH RACE 104 E 137 + MYCELIUM PRESENT WITHOUT & ⊕ WITH NECROSIS. ● PUSTULES PRESENT SCALE : ⊢—⊣ 1 MM.

In the O reaction, necrosis is less intense. A lower proportion of cells become necrotic (Plate I.7), runner hyphae were observed, and greater spread of mycelium occurs than in the OO reaction type. Growth of the colony is slow, however, and the pathogen is confined (Figs. II.2b and II.3b). The dense mycelial mat associated with spore production is not achieved, and although a level of tolerance is maintained, spore production is unable to take place.

Where chlorosis occurs, as it often does in association with the O reaction, and occasionally in association with susceptible reactions, the mesophyll cell contents become disorganised, and cell walls break down to give a granular mass. There appears to be no noticeable adverse effect on the fungal mycelium when present in a susceptible reaction, though reduced growth resulting from insufficient nutrients would be expected.

No haustoria have been observed in the resistant reactions, but the effect of cell collapse obscured all internal cell detail. It is possible that in the O reaction, haustorial connections could result in necrosis and cell collapse, which would only affect certain cells.

Discussion

The observations of hyphal fusions (Plate II.5b) indicate that nuclear exchange between mycelial strands within a leaf is possible, and that nuclear reassortment is plausible. This would, however, be dependent on colonies of two different races coming into contact: the extent to which individual colonies are able to spread would therefore influence the likelihood of such contact being established.

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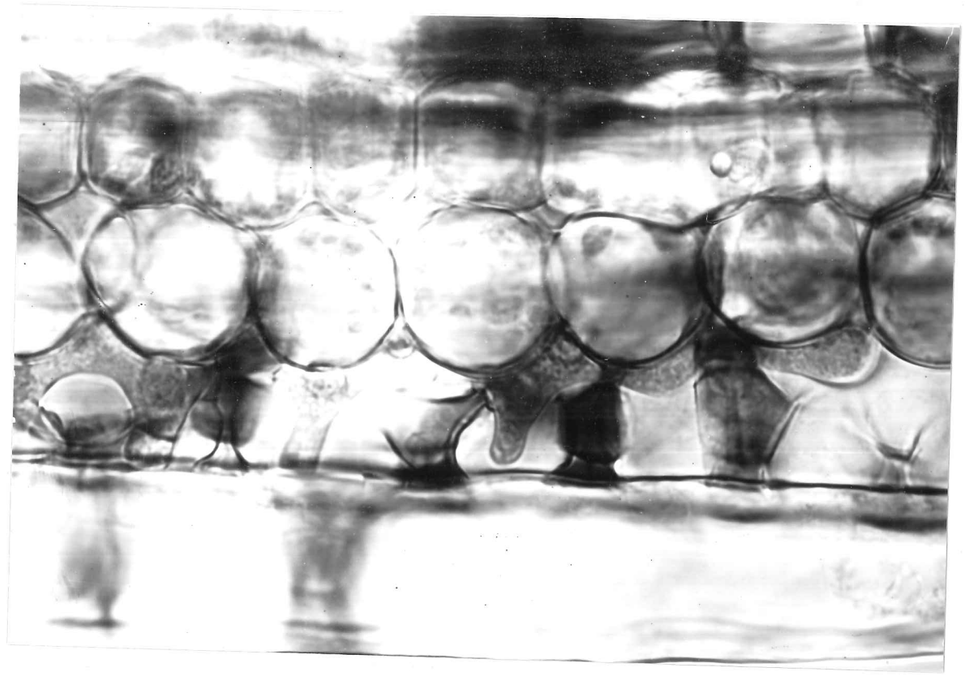
Plate II.7. Necrosis and cell collapse associated with a O reaction. Not all cells in contact with mycelium affected. (x 1000).



White (1964) suggested that the amount of cell necrosis was indicative of the intensity of the resistance exhibited, but Ogle and Brown (1971) found that the largest areas of necrotic tissue was associated with intermediate reactions, and similar observations have been recorded here. The 00 reaction gives a very intense resistance, which confines the pathogen and associated necrosis to a very small area; the 0 reaction, which is comparable to an intermediate reaction, is a less intense resistance, in which necrosis does not affect all the cells, but the total area exhibiting necrosis is larger (Table II.4). In the susceptible reaction, necrosis is scarce, and mycelial spread is extensive.

Table II.4. Relative areas of necrotic leaf tissue of varieties inoculated with races 104 E 137 and 41 E 136 of P. striiformis

Variety	Race	Reaction	Area mm^2
Heine 110	104 E 137	00	1.0
Hybrid 46	41 E 136	0	22.5
Maris Envoy	104 E 137	00	2.3
Heines Kolben	104 E 137	0	21.0

This is in agreement with Stakman's (1915) work, that the more resistant a host is, the more rapid and complete is the collapse of the host cells and the sooner the fungus becomes inactive.

Leath and Rowell (1966) illustrated the inability of P. graminis to produce haustoria in immune host varieties, but Rice (1927) had suggested that the pathogen was not solely dependent on the production of haustoria for obtaining nutrients as she observed extensive mycelial development prior to the formation of haustoria. In this work, haustoria were only observed from the infection hyphae, and none were observed in resistant reactions. It is possible that the presence of haustorial connections in the 0 type of reaction resulted in the collapse of the individual cells involved, rather than the complete necrosis of the 00 reaction. The complete necrosis exhibited in the 00 reaction is likely

to be the result of "contact inhibition" due to the diffusable substances being produced by infected cells, causing nearby cells to collapse, and thus limiting the growth of the pathogen.

Caten and Jinks (1966) outlined several methods by which heterokaryosis could arise, including 'forced heterokaryons' which had arisen under a heavy selection pressure and were unable to grow in less exacting conditions. They suggested that they would be unlikely to arise in nature, as the two homokaryons would have to meet and anastomose in favourable conditions, and then be subjected to conditions which allowed only the heterokaryon to grow.

From this work, the 0 type of reaction would offer these conditions, as the mycelium, although limited by resistance, is able to grow, although spore formation is restricted. If two or more races were present, the possibility of overlapping colonies would be likely in most leaves, which would bring the two mycelial types together. If anastomosis took place, as has been observed, a more virulent recombinant may be able to achieve spore formation, thus over coming the limiting resistance affecting the two parental races.

In the 00 reaction, the colonies are quickly confined, and a very high infection level of approximately one penetration per mm^2 would be necessary to bring the two mycelial types together. If a host is susceptible to two races present recombination is likely, but only recombinants as competitive as the two parental races are likely to be detected. A recombinant from this or the 0 reaction may well contain the necessary virulence to overcome the 00 type of resistance exhibited by another host.

The best chance of contact between colonies occurs when two races giving susceptible infection types are grown together, or possibly when a race giving a susceptible infection type and one giving an intermediately resistant (0) type are grown together. These conditions have both yielded recombinant isolates in this work: MN-7; MB-5 and MB-6.

It is possible that contact between the colonies will occur only in the stages immediately preceding sporulation, when the spore initiating hyphae are well established. Spore initiation appears to be related to the density of the mycelium in the tissue, and is probably an indirect result of the reduction in available nutrients.

Hyphal fusions were not observed among the mesophyll cells, though this could have been due either to their absence, or to the poor visibility resulting from the denseness of the leaf tissue in these areas. If these fusions are typically located at the tips of the spore initiating hyphae, recombinants would not be evident in the form of more vigorous growth or sporulation and would only be detected in isolated spores in the next generation. The selection of more virulent recombinants might be quite simple using judiciously chosen hosts, e.g. Hybrid 46 and Heine 110 selections. However, selections of less virulent combinations would be more difficult.

Although varietal variation was observed in both percentage germination and penetration frequency, this was found to have little influence on the development of colonies, as germination and penetration frequency appeared to vary independently of reaction type. These observations support those of Zadoks (1966) on P. recondita and Brown and Shipton (1964) on P. graminis tritici that the number of pustules per unit area and that reaction type were not necessarily related to the number of penetration points established.

III. CYTOLOGICAL STUDIES

III1. Literature Review

1a. Introduction

Most of the investigations of variation and physiologic specialisation in the rusts concentrate on the genetic aspect of the problem, and propose possible methods by which this variation could arise from the number of new races obtained. Few workers have considered the cytology, though Little and Manners (1969b) and Konovalova and Schektokova (1970) supported their work in this way. They showed germ tube fusions and stained germ tube and spore nuclei, but were unable to observe nuclear exchange or chromosome number.

Cytological observations are needed to support the observations in the first section, and could provide evidence to favour certain methods of asexual reproduction.

1b. Factors affecting germination in P. striiformis

Yarwood in his review of obligate parasitism (1956) suggested that the spores of obligate parasites would readily germinate, but that the germ tube would only grow as long as the food supply was not limiting.

Germination is more sensitive to environmental conditions in P. striiformis than in most other rusts. Hassebrauk and Schroeder (1966) suggested that spores produced during periods of cyclonic weather germinated more slowly and less intensely than those produced during anticyclonic weather, though these conditions were thought not to affect spores produced in the glasshouse or growth chambers. Sharp (1967) described a relationship between low germination and low wind recordings: he suggested that in these conditions there is an increase in the

concentration of large ions in the air, which reduce germination. Johnson et al. (1970) illustrated a weak negative correlation between germination and atmospheric sulphur dioxide. Fuchs (1972) also commented on the variability of infection levels obtained from inoculations.

Strobel (1965) showed that hydration of uredospores of P. striiformis greatly increased germination, and suggested that it removes an exogenous inhibitor. He showed that at least two soluble protein components change as the result of hydration, and that the liquid globules become dispersed. He also showed that the mitochondria become re-aligned about the long axis of the spores, thus increasing oxygen uptake. The breakdown of storage compounds also preceded germination. It is possible that separate hydration of spores prior to inoculation is unnecessary, as the high relative humidity during the incubation period, 90-100% (Manners, 1950) should be sufficient to hydrate the spores as part of the inoculation process on host plants or artificial media.

Spore density affects germination (Manners, 1950). Tollenaar and Houston (1965, 1966b) working on P. graminis and P. striiformis have shown that an increase in spore density can be correlated with an increase in germination, to a certain level, after which there was no noticeable self inhibition or stimulation.

Scott et al. (pers. comm.) found that when fresh spores were seeded onto plates on which spores had previously been germinated on cellophane sheets, better germination was obtained. This would indicate the presence of a diffusable self stimulator, which would account for the increase in germination with spore density.

A number of workers have found that the spores of many fungi germinate poorly if they are too closely packed: the term 'self inhibition' has been given to this phenomenon. Hassebrauk and Schroeder (1966) managed to extract the inhibitor from P. striiformis, and they have shown that the amount of germination was inversely related to the concentration of the inhibitor. It would therefore seem that there is a critical balance between the concentration of the self stimulator and the self inhibitor produced, and this would account for the threshold level of germination at a certain spore density.

The presence of a self inhibitor in P. graminis and other rust fungi has frequently been reported (Allen, 1955, 1965; Syamananda and Staples, 1961; Maheshwari and Susman, 1970) but has recently been identified in P. striiformis as methyl ferulate, (Macko et al., 1971). Maheshwari et al. (1967) and Schipper et al. (1969) suggested methods by which the germination inhibitor could be removed, resulting in synchronous germination. French and Weintraub (1957) established that nonanal and related compounds stimulated germination, as do the terpenes (French, 1961).

Tollenaar and Houston (1966a) have shown a correlation between light and temperature on germination of P. striiformis showing that light inhibited germination at 6°C but promoted germination at 11°C, when spores were produced at 0.5-4.5°C. A 15°C pretreatment reduced the light inhibition effect at 6°C, and spores exposed to light at 15°C prior to germination gave the highest germination.

McCraken and Burleigh (1962) germinated spores of P. striiformis at 2, 5, 13.5, 15 and 20°C for 15 to 20 hours under fluorescent lights and in the dark. They also used red, blue, yellow and green filters at 2° and 13.5°C. They found that germination was higher at 2° and 5°C,

and compared to 13.5°C, there was no significant difference between the presence or absence of full or filtered light. At 13.5°C however, unfiltered and blue light gave better germination than in the dark. This would suggest that germination is not light sensitive at low temperatures, but becomes so in the 10-15°C range.

It has been reported that best germination has been obtained at temperatures of less than 15°C, as the drop in temperature increases the humidity and dew formation is induced, thus improving germination (Hassebrauk and Schroeder, 1966). Chamberlain et al. (1970) used 7°C for inoculation, and Zadoks and Rijsdijk (1972) suggested that 4°C is the lower limit for germination, although Sharp (1966) obtained germination at 2°C, and Johnson (pers. comm.) has obtained germination at temperatures of 2°C and lower.

1c. Fusion bodies and nuclear exchange

Rodenhiser and Hurd-Karrar (1947), Manners (1950), and Staples and Wynn (1965) have reported fusion bodies among germ tubes on artificial media, and Manners (1950) also noted the formation of secondary vesicles, which produced one or two hyphae which were thicker walled than the original germ tubes. He suggested that they were similar to the bodies observed by Straib (1940) and that they could be homologous with substomatal vesicles. Rodenhiser and Hurd-Karrar reported the fusions as rounded bodies at the hyphal tips which fused with neighbouring hyphae, whereas Staples and Wynn (1965) reported that the fusing aerial germ tube tips became liquid and then solidified. Konovalova and Shekotkova (1970) classified four types of fusion bodies in P. striiformis: Type 1 - rounded bodies with a pellucid membrane and granular contents, similar to uredospores in colour; Type 2 - rounded bodies lacking the thick membrane, but similar in colour to

type 1; Type 3 - in the form of hyphal thickenings; Type 4 - like a glomus of fusing hyphae. Wilcoxson et al. (1958) reported anastomoses between germ tubes of 9 isolates of P. graminis on 7 different media. They found that mineral oil permitted germination, but reduced anastomosis and suppressed fusion body formation, but higher concentrations of sucrose in the agar appeared to stimulate fusion body production, and they were able to detect fusion body nuclei, though these have not yet been observed in P. striiformis. Little (1966) and Little and Manners (1969b) reported germ tube fusion and the passage of cytoplasm between germ tubes of P. striiformis but provided no evidence for nuclear exchange.

Nuclear exchange between races of P. striiformis is difficult to demonstrate cytologically especially as until recently (Brown and Sharp, 1970) no colour mutants have been reported and different races cannot be distinguished under the microscope, even using electron microscope techniques (Stanbridge and Gay, 1969). A method of identifying races cytologically would greatly facilitate studies of nuclear exchange. A possible method by which this could be achieved is to use radioactive labels to label spore nuclei.

Ehrlich and Ehrlich (1970) used ^{14}Carbon to study the transfer of carbon from the labelled pathogen, P. graminis to the host cells. They inoculated wheat seedlings with spores labelled with ^{14}Carbon, and showed the passage of radioactivity from the pathogen to the host chloroplasts using electron microscope and autoradiography techniques.

In microautoradiography studies of mildew conidia, Shaw and Samborski (1956) showed that the mycelium and developing conidia took up the ^{14}Carbon, whereas the mature spores did not, and it would seem likely that this would also hold true for the rusts. Venitt (1965)

was able to detect fusions between strains 3- and 13+ using tritiated uridine in his studies of nucleic acid metabolism in Protomyces inundatus.

Tritiated thymidine has been widely used for labelling cells synthesising DNA (Baserga and Kiseleski, 1963). Taylor (1958) found that daughter chromosomes resulting from duplication in the presence of ^{3}H-Thymidine were equally and uniformly labelled, but that in ensuing duplications in the absence of the labelled precursor the label appeared in only one of the two daughter chromosomes. This suggests that the DNA is synthesised as a unit extending throughout the length of the chromosomes, the units remaining intact through succeeding replications. Woods and Schairer (1959) suggested, however, that the newly synthesised DNA is shared equally between daughter chromosomes. In one case (Woods and Schairer, 1959) all the daughter chromosomes would contain some labelled DNA, in the other (Taylor, 1958), only half of the daughter chromosomes would contain labelled DNA. This would mean that it would be quite possible for some daughter nuclei not to contain any labelled DNA.

1d. Staining of nuclei

A number of nuclear stains have been used successfully with fungi. Beckett and Wilson (1968) used a propionic carmine technique for asci of Podospora anserina, and were able to follow the cytology through karyogamy, three nuclear divisions in the ascus, the first spore mitosis and maturation. Feulgen has been used on Penicillium (Fjeld and Laane, 1970), and propionic haematoxylin on the Ascomycete Gelasinospora calospora (Lu, 1967). Laane (1970) has used Giemsa-HCl successfully on Penicillium, and it has also been used on Pellicularia koleroga (Finley, 1970), Ophiobolus graminis (Chambers, 1970) and P. graminis (Williams and Hartley, 1971). Acetic orcein has been used successfully on Penicillium (Laane, 1967 and 1969).

Few cytological studies have been reported on the rusts, but Kapooria (1968) has shown that the nuclei in the promycelium of Puccinia pennisetti contain 5 chromosomes, two pairs and a solitary one. Mc Ginnis (1953) used acetic orcein and showed that the chromosomes in P. graminis teliospores formed a continuous ring or spireme during the first nuclear division. He established the chromosome number as n = 6. He suggested that stem rust is a polyploid, as the 6 chromosomes appear as three associations of two chromosomes. He raised the question whether the polyploid nature of the rust had any bearing on increased pathogenicity or wider host range, as it has frequently been observed that polyploids are better adapted to more varied conditions than are diploids.

In 1954 Mc Ginnis observed the chromosomes of P. coronata and established 3 as the haploid number, supporting the polyploid hypothesis, with 3 representing the basic chromosome number for the genus. By extending his work in 1956 to further Puccinia species, he offered two basic chromosome numbers for the genus, 2 and 3. He suggested that the chromosome number may control sexual behaviour, as of the species he studied, all the homothallic species had n = 4, and all the heterothallic species, n = 6. Williams and Hartley (1971) reported one axenic rust colony of P. graminis to have 12 chromosomes, and suggested the colony was diploid.

Little and Manners (1969b) have shown the spores of P. striiformis to be binucleate, using Flemming's Triple stain, with occasional incidents of uni- and tri-nucleate spores. They reported the smallest diameter of the nuclei to range from 0.5 μ to 1.0 μ with a mean of 0.7 μ. They used Heidenhain's haematoxylin to stain germ tube nuclei, and showed that the two nuclei were frequently near the tips of the germ tubes.

Little (1966) found that stains involving hydrolysis were too drastic for staining nuclei of P. striiformis, as the spore and germ tube walls disintegrated before the hydrolysis was completed.

Valkoun and Bartos (1972) have obtained a chromosome count for P. recondita, using the staining method of Lu (1962). They suggest that there are 6 chromosomes, ranging from 0.5-1.2 μ. Twelve chromosomes were counted at early anaphase figures and he suggests that the division is basically similar to the mitotic division of higher plants. He reports the presence of centrosome-like structures: these are associated with the spindle and their position is related to the orientation of the division. Although common in animals and flagellate plants, they are uncommon in higher plants.

1e. Interference and phase contrast techniques

Aist and Wilson (1968) used phase contrast techniques to compare Giemsa-HCl preparations with living material of Ceratocystis fagacearum and Fusarium oxysporum. They suggested that many disputed aspects of nuclear division could be resolved by observations on the living fungal cell, using phase contrast. Kimber and Wolfe (1966) used interference contrast to observe the two chromosomes of Erysiphe graminis, though it has been suggested that the two 'chromosomes' observed could be chromosomal aggregations, sometimes observed prior to nuclear division.

1f. Factors affecting axenic culture

The rusts have long been considered obligate parasites, implying that they had not been successfully grown on artificial media, free of the living host. However, Williams et al. (1966) obtained vegetative growth of Puccinia graminis f. sp. tritici in axenic culture, and later reported that the cultures sporulated and were binucleate and pathogenic

(Williams et al., 1967). The concept of obligate parasitism since these achievements is discussed by Scott and Maclean (1969). More recently other workers have successfully grown Australian (Bushnell, 1968; Wong and Willetts, 1970), American (Bushnell and Stewart, 1971) and British (Maclean, pers. comm.) isolates of P. graminis in axenic culture.

A number of other rusts have been added to the list: P. recondita (Singleton and Young, 1968); P. graminis f. sp. avenea, (Scott and Maclean, 1969); P. graminis f. sp. secalis and P. coronata f. sp. avenae (Kuhl et al., 1971); Melamspora lini (Turel, 1969).

P. striiformis has not yet been grown in axenic culture and it was felt that in order to obtain mycelium free of host tissue, to facilitate the cytological studies, attempts should be made to grow P. striiformis in pure culture. Young cultures of P. graminis have been used by Williams et al. (1966) and Williams and Hartley (1971) to make cytological studies of nuclear and chromosome number.

The physiology of the obligate parasites has been extensively considered. Dickinson (1949a, b, c, d, 1955, 1969, 1970 and 1971) has given much attention to the necessary conditions for germination, and the fungal response to physical stimuli. He considered the presence of a cuticular membrane to be essential for growth, but Chakravorti (1966) showed that P. graminis formed appressoria even in sub-epidermal inoculations, illustrating that the cuticular membrane is not essential for infection, but that the germ tube's transformation to infection hyphae will only occur after the formation of an appressorium and vesicle. Woodbury and Stahman (1970) considered the association of spore germination with materials forming films of water on their surfaces. They suggested that the amount of film formed, may be correlated with the

wettability of the host cuticle and the ability to differentiate infection structures *in vitro* or on artificial membranes. They also suggested that the surface film might interact with the host cuticle to determine the size and configuration of infection droplets, thus providing a surface of defined area on which film reactions occur, resulting in the sequence of products controlling germination, direction of growth and differentiation of infection structures.

Loss of metabolites from rust spores and mycelium to the external medium during germination and in axenic cultures has been reported (Daly *et al.*, 1967; Jones and Snow, 1965; Staples and Wynn, 1965), and described as 'leakiness'. Daly *et al.* (1967) used ^{14}Carbon labelled uredospores and showed that approximately 7% of the total spore carbon was lost to the medium during the early stages of germination. At the end of the 3rd hour, amounts of carbohydrate external to the sporelings was greater than the amount of carbohydrate within the sporelings. Since external amounts of carbohydrate subsequently decreased, these workers suggested that they were taken up again for subsequent metabolism. These results illustrate that essential metabolites are lost to the external medium during germination, and this may account in part for the failure of many germ tubes to initiate saprophytic growth, especially in regions of low inoculum density. Leaky hyphae would be an advantage in the host tissue, as nutrient absorption would be possible through the hyphal walls as well as through the haustoria. This would give support to Rice's observation (1927), that hyphal growth is able to precede the formation of haustoria.

Brian (1967) indicated that no net protein synthesis takes place during spore germination, and that any new molecules formed are mainly the result of turnover at the expense of existing protein. DNA and RNA synthesis is similarly restricted.

Wong and Willetts (1970) found that P. graminis grew better on gelatin than on agar, and only in the presence of Evans peptone. They also suggested that bovine serum reduced the 'leakiness' of the fungus, causing it to retain essential nutrients for growth. Bushnell and Stewart (1971) seeded their plates with spores suspended in distilled water. This was placed in glass rings in the previously dried agar. They found that agar absorbed the moisture, and the higher spore concentrations obtained improved germination.

Williams et al. (1966, 1967) obtained successful cultures when the spores were seeded onto a medium containing Czapek's minerals and yeast extract. The addition of Evans peptone improved the medium, and sporulation was reported (Williams et al., 1967). The cultures were pathogenic if applied to the leaf mesophyll, though they failed to penetrate the epidermis.

1g. Problems of asepsis in axenic culture

Yarwood (1956) outlined some of the problems of asepsis in culturing work, especially in connection with the parasitic fungi. Ramakrishnan and Staples (1970) have shown that the antibiotic Actinomycin D, which inhibits RNA polymerase, blocks the differentiation of rust uredospores, and suggested that the formation of infection structures is necessary for infection, and may accompany or depend on the synthesis of RNA. Brian (1967) reported that Streptomycin also causes cessation of protein synthesis and prevents sporeling growth in some fungi.

Williams et al. (1966, 1967) obtained uncontaminated uredospores of P. graminis by harvesting infected leaves at the fleck stage, surface sterilising them for 4 minutes in 15% Calsol containing 0.001% Tween 80 and washing the leaves in 3 changes of distilled water. The leaves were

then placed on nutrient agar over calcium chloride at 17-18°C with 400 ft. candles light for 4-5 days, by which time sporulation had taken place.

III2. Experimental Methods and Materials

2i) Cytological studies and chromosome counts

2ia. Slide preparation for uredospore and germ tube observations.

It was necessary to find a medium which would allow spore germination, adhere the material to the slides and not retain the cytological dyes used.

Varying concentrations of agar and gelatin retained the dyes at levels which were unacceptable for these studies, and Mayers albumen frequently became granular during staining procedures, thus interfering with observations. Following overnight incubation of P. striiformis spores at 7°C, germination levels of 85% were obtained on distilled water in petri dishes, compared with less than 5% on water droplets on glass slides. However, the plastic petri dishes were not optically suitable for microscopical observations.

Germination chambers were prepared by sticking Van Teighem rings on to microscope slides using paraffin wax. It was found that germination in these chambers equalled and sometimes exceeded that obtained in the petri dishes.

After germination had been assessed, the water from each chamber was removed using a hypodermic syringe, and the slides were placed on a hot plate. This served two purposes: to melt the wax in order to remove the van Teighem rings; and to adhere the spores and germ tubes to the slides. The preparations were then ready for fixation and staining.

2ib. Fixatives.

The choice of fixative is complex as it is necessary to penetrate the resistant spore wall and preserve the spore contents with minimum distortion. Four fixatives were used: Carnoy's fixative; Schaudinn's fixative ($\frac{1}{2}$ strength) plus 2% glacial acetic acid; Singh's (1969) fixative; and the modified fixative after Lu and Raju (1970).

Carnoy's and Singh's fixatives shrink the chromosomes and cytoplasm, and can reduce the effectiveness of some of the aqueous stains. Carnoy's fixative was used most frequently in this work, however, mainly in conjunction with the Giemsa-HCl staining technique.

Schaudinn's fixative was used exclusively with the Heidenhain's haematoxylin staining method. It did not shrink the cell contents as much as Carnoy's fixative, but the staining method was not adopted for this work. Material could not be stored in the BAC fixative of Lu and Raju, as it contains chromic acid which macerates the tissues and caused total disintegration of the tissue if left in the mixture for more than 5 days.

2ic. Leaf section preparations.

Infected sporulating leaves were cut into lengths of about 1 cm and fixed in Carnoy's fixative for 24 hrs. and then transferred to absolute alcohol for at least 12 hours to complete the dehydration. The leaves were transferred to chloroform through an alcohol/chloroform series, and when in chloroform paraffin wax was added gradually over a period of 2 days at 30°C until no smell of chloroform remained. The wax was replaced with fresh wax and the leaf segment was transferred to

an embedding dish and the wax cooled quickly. Thin sections (8-10 μm) were cut on a Cambridge Rocker Microtome. The ribboned sections were placed on microscope slides with a thin layer of albumen and dried for 24 hours.

The wax was removed by passing the slides through 2 changes of xylol, and the slides were transferred to 70% alcohol for storing. The sections were stained using the stains listed below.

2id. Stains.

A number of staining techniques were tried, including Giemsa-HCl, Propionic Iron Haematoxylin, Mayer's Haemalum, Flemming's Triple Stain, Heidenhain's haematoxylin, Conant's Quadruple stain, Acetic Orcein, Acridine Orange, Singh's (1969) Propionic Carmine, Aceto carmine, Methyl Green Pyronin, and Saffranin and Fast Green. In all methods except Conant's Quadruple stain and Saffranin and Fast Green, nuclei were detectable, but Giemsa-HCl and Propionic Iron Haematoxylin were most frequently used as these stains also differentiated the chromosomes within the nuclei.

Good results were obtained using Flemming's Triple stain and Mayers Haemalum with leaf sections.

2ie. Electron Micrograph preparations[1]

Infected sporulating leaves were cut into 2 x 2 mm pieces and fixed for ½-1 hour in 4% Gluteraldehyde in 0.1 ml phosphate buffer (pH 7.0) with 2% sucrose added. They were washed in buffer and transferred to 1% Osmic acid in phosphate buffer for ½-1 hour, then

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[1] My thanks to Dr. P. Wooding of the A.R.C. Babraham for his help in the preparation and interpretation of these sections.

dehydrated through an alcohol series finishing with 2 changes of absolute alcohol, and then placed in Propylene Oxide for 15 minutes. The pieces were transferred through a propylene oxide/resin mixture to 100% resin over a period of 36 hours and placed in embedding boats, then left at 60°C overnight and finally raised to 100°C and allowed to cool.

Loose spores were also embedded, but these were placed in 1% agar before fixation as this facilitated handling. Some pieces of leaf were also fixed in a Potassium permanganate fixative. This destroys the nucleus and nucleolus, leaving only the nuclear membrane.

Embedded material was sectioned on a L.K.B. Ultratome 1 microtome and examined with an A.E.I. EM. XB. electron microscope.

2i f. Observations.

Light microscope observations were made using a Vickers M15c microscope. All nuclear observations and measurements were made at a magnification of x 1350, using a x 90 oil immersion lens. Photographs were taken with a 35 mm camera with automatic exposure meter.

Nuclear number, nuclear and nucleolar size and chromosome number were assessed. Observations of unstained fused germ tubes were made and the number of nuclei in fusion bodies was also studied from stained preparations.

Teleutospores of race 41 E 136 and 104 E 137 were collected from field plots, in order to examine the nuclei and determine the size relative to the uredospore nuclei. Teleutospores were scraped off the leaves and gently squashed in distilled water. Observations were made at x 600, using a Watson Microsystem 70 Phase contrast system, as the nuclei were easily visible with this method (Plate III.1a) and obviated staining.

2ii) Radioactive labelling of P. striiformis

2iia. Introduction.

As races of P. striiformis are indistinguishable microscopically, it was necessary to introduce a label to one of the races in order to detect whether nuclear exchange was taking place through the germ tube fusions observed.

As light microscope techniques were to be used, an isotope with short emission was required to give high resolution autoradiography. Tritium (^{3}H) is a useful biological tracer: its low toxicity places it in the lowest class of radioactive materials, and no shielding precautions are necessary. The half life is 12.25 years (Wilson Ed. 1966). Tritium compounds give rise to very soft β emissions which only travel 6 mm in air, and 1 μm in photographic emulsions, (Evans, 1966). This gives the advantages of high resolution autoradiography, as it permits the precise location of the activity. These properties make ^{3}H more suitable than ^{14}C for experiments in which it was desired to distinguish between unlabelled and labelled nuclei which might be close to each other.

Observation of nuclear exchange was required, so isotopically labelled thymidine, a specific precursor of DNA which has been extensively used (Taylor et al., 1957; Painter et al., 1958; Firket and Verly, 1958; Davidson, 1965) was chosen.

If ^{3}H-Thymidine can be applied during spore formation and DNA synthesis, spores with labelled nuclei should be produced and should therefore be identifiable using autoradiographic techniques. If labelled spores were germinated together with unlabelled spores, one should be able to distinguish between the two spore types.

Thymidine-6-H3 was obtained from the Radiochemical Centre, Amersham, at a concentration of 1 mCi/ml and stored at 0°C until required. As more than 95% of the ^{3}H was present at the 6-position, from which it is not easily lost, it did not deteriorate noticeably with storage.

Ilford nuclear research emulsion type K2 was used for the preparation of autoradiographs.

2iib. Experiment I.

A preliminary experiment was prepared, in which well sporulating leaves of the winter wheat variety Jubilegem were placed in distilled water containing ^{3}H-Thymidine at concentrations of 10 and 20 μCi/ml. The spores from the leaves were collected daily, by brushing them into specimen tubes containing distilled water, and stored at 0°C.

After 4 days, when sporulation had ceased, the collected spores were centrifuged at 6,000 r.p.m. for 2 mins., then washed and recentrifuged twice. The spores were then treated with 0.4 ml of 5% Perchloric acid at 60°C for 30 mins., to extract the DNA. When cold the samples were centrifuged again and 0.2 ml of the supernatant was placed in 10 ml of scintillation fluid containing PPO 4 g, POPOP 0.2 g, Toluene 667 ml, Triton X-100 333 ml. As Triton X-100 mixtures exhibit unstable phase systems at certain water concentrations, a constant amount of distilled water was added to clear the turbidity in the samples. The samples were then counted in a Tracerlab Coru/matic 200 scintillation counter.

The results obtained showed no significant level of ^{3}H in any of the samples, so further experiments were designed to determine the best time and methods of applying the label to ensure that the spore nuclei became labelled.

2iic. Experiment II

Seeds of Jubilegem were sown in vermiculite and watered with Hoaglands culture solution (Appendix II). After 10 days they were inoculated with race 41 E 136. Two days later, individual plants were carefully placed in plastic thimbles with holes of 3 mm diameter in the bottom, which were supported by a black perspex rack over a tray containing Hoaglands culture solution. Air was pumped into the culture solution to aerate it, and the whole unit was covered with an isolation chamber to prevent contamination of the glasshouse compartment in which the plants were grown (Fig. III.1).

At intervals of approximately 24 hours, five plants in their thimbles were transferred from the culture solution to a beaker containing a solution of 20 μm/ml ^{3}H-Thymidine in distilled water and left for 24 hours. The thimbles were supported on wire mesh, and the netting and beaker were covered with aluminium foil to keep out the light. A lamp glass with cellophane over the top was placed over the plants to prevent spore loss and contamination of the glasshouse compartment (Fig. III.2).

The roots of the labelled plants were washed thoroughly in distilled water and the plants were returned to their places in the culture solution. Unlabelled controls were kept in the same container of culture solution so that any contamination of the culture solution could be detected and allowed for. Temperature and dosing time were recorded and the onset of flecking and sporulation was noted.

Spores were collected from each treatment 14 days after inoculation, and germinated on 1% water agar containing 0.5% glucose. The plants were harvested 4 days later. In each treatment the four leaves were kept separate, and the roots and leaves were dried at 100°C overnight. The

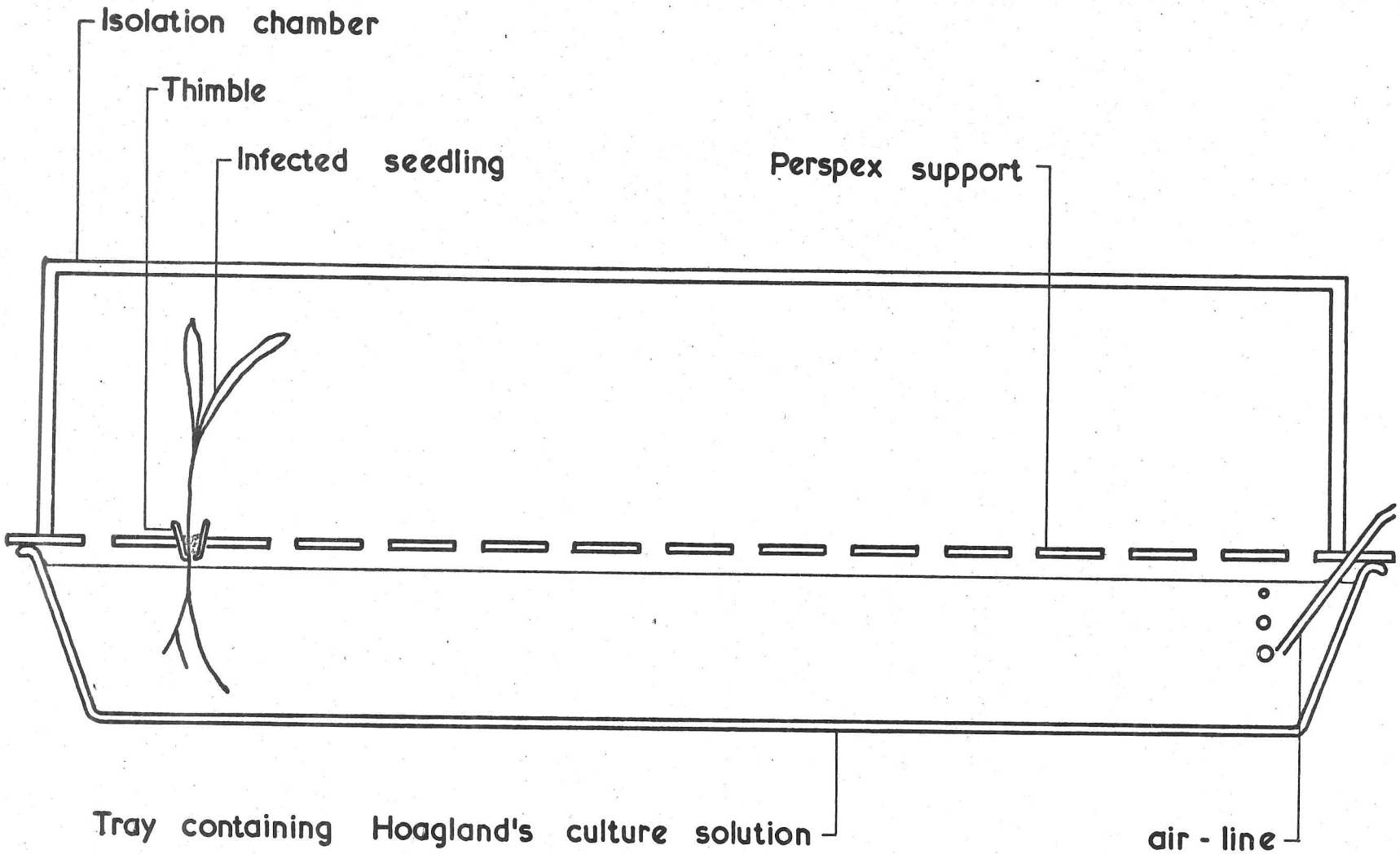


FIG III.1 Diagram of Apparatus for R.A. Experiment 2.

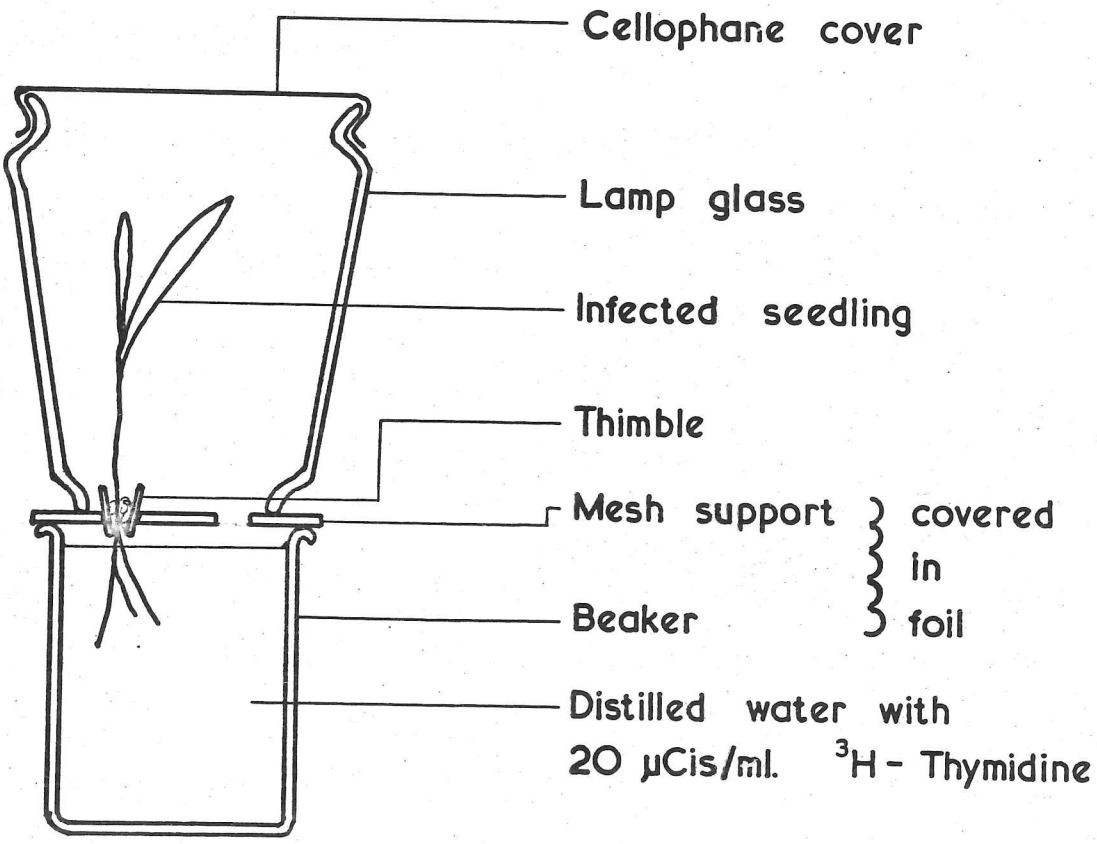


FIG III.2. Apparatus used for applying ^{3}H-Thymidine in R.A. Experiment 2.

dry weight of each sample was recorded. Each sample was treated with 5 ml of 5% Perchloric acid at 60°C for 1 hour, macerated, and then 2 ml of the suspension was withdrawn. This suspension was centrifuged at 6,000 r.p.m. for 2 mins. and 0.1 ml of the supernatant was added to the scintillation fluid as above. The samples were then counted and corrected for counting efficiency and quenching using the external standards channel ratio method (Tracerlab Information Bulletin, 1971; Takahashi and Blanchard, 1970).

The plants had suffered considerable mechanical damage during the transfer of individual plants to and from the ^{3}H-Thymidine solution, so the experiment was modified.

2iid. Experiment III

Seeds of the variety PA 325 were germinated on filter papers in the laboratory. After 6 days, the seedlings were placed in thimbles as above. Three inch (7.5 cm) pots were modified to support the thimbles by putting four holes of diameter 1.7 mm in the bottom. The pot was supported in a perspex sheet over the aerated culture solution as above, giving a compact, handleable unit containing the four plants. Each unit was again covered with a lamp glass and cellophane (Fig. III.3). This meant that the individual plants were only handled after sporulation had started.

Two days after transferring the seedlings to the thimbles when the seedling leaves had fully expanded, the seedlings from two treatments were inoculated with race 104 E 137 by dusting talc and spore mixtures on to the plants, covering them with lamp glasses and polythene tops. All four treatments were put in the refrigerator overnight. The trays of plants were returned to the glasshouse the next day, and the cellophane tops were replaced.

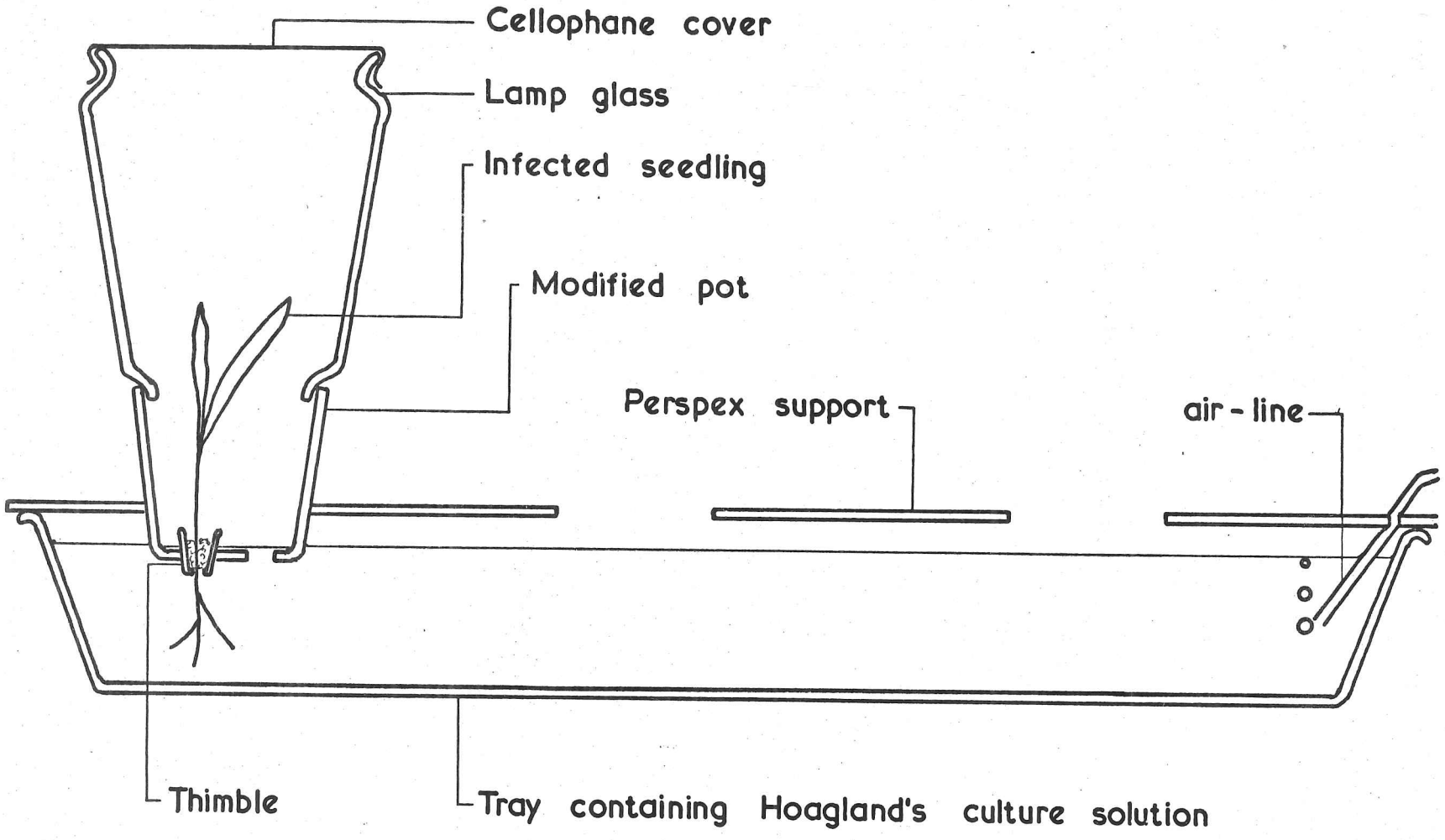


FIG III.3. Diagram of Apparatus for R.A. Experiment 3.

On the next and each of the nine following days, one pot of inoculated and one of uninoculated plants were put into a beaker containing distilled water and 20 μCi/ml ^{3}H-Thymidine, and one pot of uninoculated and one pot of inoculated plants were placed in beakers containing distilled water only. They were left for 24 hours and the roots were then thoroughly washed and the pots were returned to the culture solution.

Fourteen days after inoculation, there was some infection but a very low level of sporulation. The seedlings appeared etiolated, probably as a result of the high glasshouse temperatures in the summer months, and the experiment was terminated.

2iie. Experiment IV

The results of Experiment II showed (Fig. III.7 P.135) that a rapid increase in uptake of ^{3}H by the first leaf occurred at the time of development of chlorotic flecks, possibly due to the growth of the pathogen and spore formation below the epidermis. It was therefore decided to grow the plants in pots of potting compost and use the usual procedures (Ch. I.1iiid.) for inoculation. When flecking was observed, groups of 6 well infected plants were cut and placed in specimen tubes containing 4 ml of culture solution until sporulation. The culture solution was changed daily, and the plants sporulated well.

Three methods of spore collection were attempted:

i) Spores were shaken off the leaves into a clean specimen tube at each collection.

ii) The infected leaf was held in a specimen tube using a split cork.

iii) The infected leaf was held in a specimen tube using cotton wool.

In all methods the tubes were tapped sharply to dislodge the spores, but in ii) and iii) some spores had accumulated in the tube during the experiment.

2iif. Experiment V

Seedlings of Maris Beacon were inoculated with race 104 E 137 and seedlings of Maris Envoy were inoculated with race 41 E 136.

Nine days after inoculation, when flecking was visible, the plants were cut down to the first leaf, the roots removed and the stems placed in tubes of culture solution. As in Experiment IV, six plants were placed in each tube, and the leaves were held in the specimen tubes by cotton wool. The experiment was shaded with muslin to prevent overheating as a result of direct sunlight.

After 24 hours, and at 24 hour intervals during the next 4 days, all the culture solutions were changed. Each 24 hours, 4 ml of 20 μCi/ml ^{3}H-Thymidine was added to a different tube of each race, in place of the culture solution. After 24 hours, the ^{3}H-Thymidine was discarded, the specimen tube and plant stems were rinsed and the culture solution was replaced. The tubes were checked morning and evening to ensure that they had not dried out, and any growth in the shoot was cut back.

Four days after placing the plants in culture solution, they were sporulating well and two days later when the labelling was completed, the spores were collected and germinated in glass chambers (cf. Ch. III.2ia) on distilled water in preparation for autoradiography. Labelled and unlabelled spores were germinated separately and together.

The plants were allowed to sporulate for a further 6 days, in order to permit a further collection of spores to be made. These spores were then germinated and prepared for autoradiography, as described below.

2iig. Preparation of autoradiographs

The germinated spores were fixed and stained in Giemsa-HCl using the techniques described above.

Ilford K2 type nuclear research emulsion was used and preparation and handling of the emulsion was carried out under Ilford 'S' 902 safelight filters.

A test tube was marked with the total and the half volume required to fill the dipping vessel, and two water baths were stabilised, one at 37°C and one at 50°C. Under safelight conditions emulsion threads were placed, using forceps, in an unmarked tube and melted at 50°C. Enough of the melted emulsion was added to the $\frac{1}{2}$ volume of distilled water in the marked tube to bring the level up to the marked full volume, and was mixed by inverting once. It was then poured into the dipping vessel and maintained at 37°C.

Slides were dipped consecutively, the emulsion being changed after each 10 slides. After dipping, the slides were held vertically to drain off surplus emulsion and then dried for about 30 min in a drying cabinet at a setting of about 17°C. They were then packed in storage boxes, sealed, labelled and stored at 2-3°C for 6 months.

Development of the autoradiographs was carried out in complete darkness using Kodak 19B developer at 20°C for 2 mins. The slides were then rinsed in distilled water and fixed in Metafix for 5 mins then washed in tap water for 5 mins, rinsed in distilled water and dried in a slide rack in a cabinet at 30°C for 2 hours.

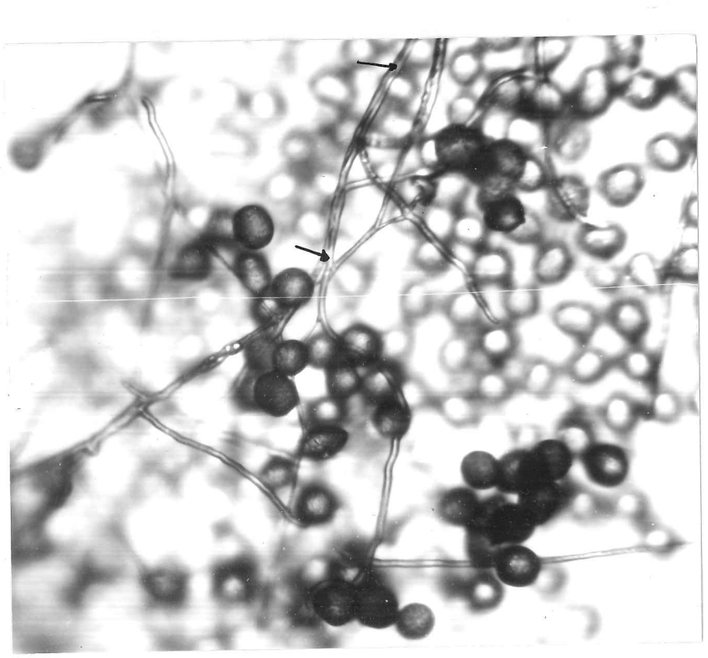
The slides were examined at a magnification of x 1,000 using an oil immersion lens.

Plate III.1a. Teleutospores. Nuclei visible. Phase contrast. (x 1000).

a

Plate III.1b. Germ tube fusions. (x 1000).

b



All contaminated non-disposable equipment was soaked and washed thoroughly in Decon 90 to remove all traces of ^{3}H.

2iii) Axenic culture of P. striiformis

2iiia. Materials and Methods

Seedlings of Michigan Amber were inoculated with spores of race 104 E 137 and 41 E 136 as described in Chapter I.1iiid. After about 7 days, when flecking was visible, the infected seedling leaves were collected and washed in 70% alcohol and then in two changes of N/10 sodium hypochlorite solution containing 0.001% Tween 80 as a wetting agent, for a total of 2 mins. The leaves were then rinsed in three changes of sterile distilled water, and placed on 2% sucrose agar slopes containing 40 ppm Benzimidazole to delay senescence in the leaf (Person et al., 1967; Doling, 1966). These were then either placed in an illuminated incubator at 10°C or under lights at room temperature (approx. 20°C), and left until the spores had ruptured the epidermis.

Three sets of culture media were prepared with Czapek minerals and Simmons Citrate agar singly and together as the basic mineral supplies. Other components of the media were added as detailed in Tables III.1, III.2, III, 3. Infected leaves were also left to senesce on the media listed in Table III.4 for 48 hours.

Table III.1. Composition of test media. I. CM1-CM6 and Sucrose

Substrates	gm/100 ml in each medium						
	CM1	CM2	CM3	CM4	CM5	CM6	Sucrose
Czapek broth	4.0	4.0	2.0	5.0	2.5	5.0	-
Agar	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Glucose	-	1.0	0.5	-	-	-	-
Sucrose	-	-	-	1.0	0.5	2.5	2.0
Evans Peptones	-	1.0	0.5	1.0	0.5	-	-
Peptonised Milk	-	-	-	-	-	0.5	-
Bacto Peptone	-	-	-	-	-	0.5	-
Yeast Extract	1.0	0.5	0.3	0.5	0.3	0.5	-
M.R.V.P. Medium (Oxoid)	0.5	-	-	-	-	-	-
Bovine Serum	-	0.3	0.1	0.2	0.1	-	-
Distilled Water	..... to 100 ml						

Table III.2. Composition of test media. II. Plates were assessed for spore germination 24 and 48 hours after inoculation, and for growth of the pathogen.

1: *** 15%
** 8-15%
* 8%

2: *** 90%
** 70-90%
* 40-70%

3: *** 10-13 days
** 8-10 days
* 6-8 days

Medium	gm/100 ml Peptone 0.1	Peptone 0.5	Peptone 1.0	Yeast Ext. 0.1	Yeast Ext. 0.5	Yeast Ext. 1.0	Serum	Percentage Germination [1]24hr	[2]48hr	[3]Growth
1	+			+				**	*	*
2	+			+			+	**	*	*
3	+				+			**	***	*
4	+				+		+	***	**	*
5	+					+		**	**	***
6	+					+	+	**	***	**
7		+		+				**	**	***
8		+		+			+	**	*	***
9		+			+			***	**	**
10		+				+		*	**	***
11		+				+	+	***	***	**
12			+	+				**	**	**
13			+	+			+	**	**	***
14			+		+			**	***	**
15			+		+		+	**	***	**
16			+			+		*	*	**
17			+			+	+	*	***	**
Basic								***	*	**

Table III.3. Composition of test media. III. Basic medium contained: 2.3% Simmons Citrate agar; 2% Sucrose; 0.5% Difco Yeast Extract.

Medium reference	Additional substrates		
	Peptones etc. 1%	Czapek Minerals 4%	Bovine Serum 0.2%
1	Bacteriological Peptone	-	-
1+	Bact. Pept.	-	+
1c	Bact. Pept.	+	-
1c+	Bact. Pept.	+	+
2	Bacto Peptone	-	-
2+	Bacto Peptone	-	+
2c	Bacto Peptone	+	-
2c+	Bacto Peptone	+	+
3	Peptonised Milk	-	-
3+	Pept. Milk	-	+
3c	Pept. Milk	+	-
3c+	Pept. Milk	+	+
4	Peptone P.	-	-
4+	Peptone P.	-	+
4c	Peptone P.	+	-
4c+	Peptone P.	+	+
5	Evans Peptone	-	-
5+	Evans Peptone	-	+
5c	Evans Peptone	+	-
5c+	Evans Peptone	+	+
6	M.R.V.P.	-	-
6+	M.R.V.P.	-	+
6c	M.R.V.P.	+	-
6c+	M.R.V.P.	+	+
7	Casamino acids	-	-
7+	Cas. acids	-	+
7c	Cas. acids	+	-
7c+	Cas. acids	+	+
8	-	+	-
8+	-	+	+

Table III.4. Percentage germination recorded on media on which leaves have been left to senesce for 48 hours.

Medium + 2% Agar	% Germination
Casamino acids	65.0
Czapek minerals	7.5
Malt	35.0
Peptone	100.0
Simmons Citrate	0.0
Water	5.0
Yeast Extract	69.0

The spores were transferred to the test media using one of two methods:

i) 2 ml of sterile distilled water was added to the slopes and shaken, to suspend the spores. 0.5 ml of this suspension was pipetted on to the agar plates.

ii) A sterile camel hair brush was used to transfer the spores from the leaf surface on to the agar plates.

All the work was carried out in a Microflow sterile air bench, using sterile equipment. Following inoculation, some plates were sealed with sellotape to prevent contamination of plates during handling.

The surface sterilised leaves produced spores at both 10°C and 20°C, 4-5 days after being treated. It was found that leaves in the 10°C lighted cabinet produced spores over a longer period of time than those kept at 20°C. This allowed further plate inoculations to be carried out at 3 day intervals.

Spore germination was assessed on all media after incubation at 7°C and 10°C for 24 and 48 hours. The plates were then transferred to a lighted 10°C cabinet, and were examined at intervals of 3 days to assess development.

Brush inoculation was found to be more suitable than the spore suspension method, as better germination and less contamination was obtained (Figs. III.4, 5).

III3. Results

3i) Cytological Studies and Chromosome Counts

3ia. Germ Tube Fusions

Germ tube fusions were observed on all media, and three distinct types were classified: germ tubes running alongside each other were seen to fuse along some sections of the area in contact (Plate III.1b). In these cases the germ tube tips frequently continued to grow independently. This sometimes resulted in the development of a meshwork of fusing germ tubes, which was the most frequently observed fusion type; alternatively, the aerial germ tube tips became swollen (Plate III.2a) and if they came into contact with another germ tube tip, fusion occurred. A single germ tube frequently continued to grow following this type of fusion; the third type of fusion occurred when a germ tube tip came in contact with the side of a second germ tube, and a fusion point was formed.

Cytoplasmic streaming was frequently observed, and in one case the cytoplasms which were close together in parallel were seen to stream down the length of these germ tubes, and the contents from both of the germ tubes to enter a third germ tube at the point of fusion.

Several nuclei were usually observed in fusion bodies, which would suggest that the nuclei were involved in cytoplasmic streaming, and where fusion bodies are formed, nuclear exchange could be assumed to take place. Seven nuclei have been observed in one fusion body (Plate III.2b).

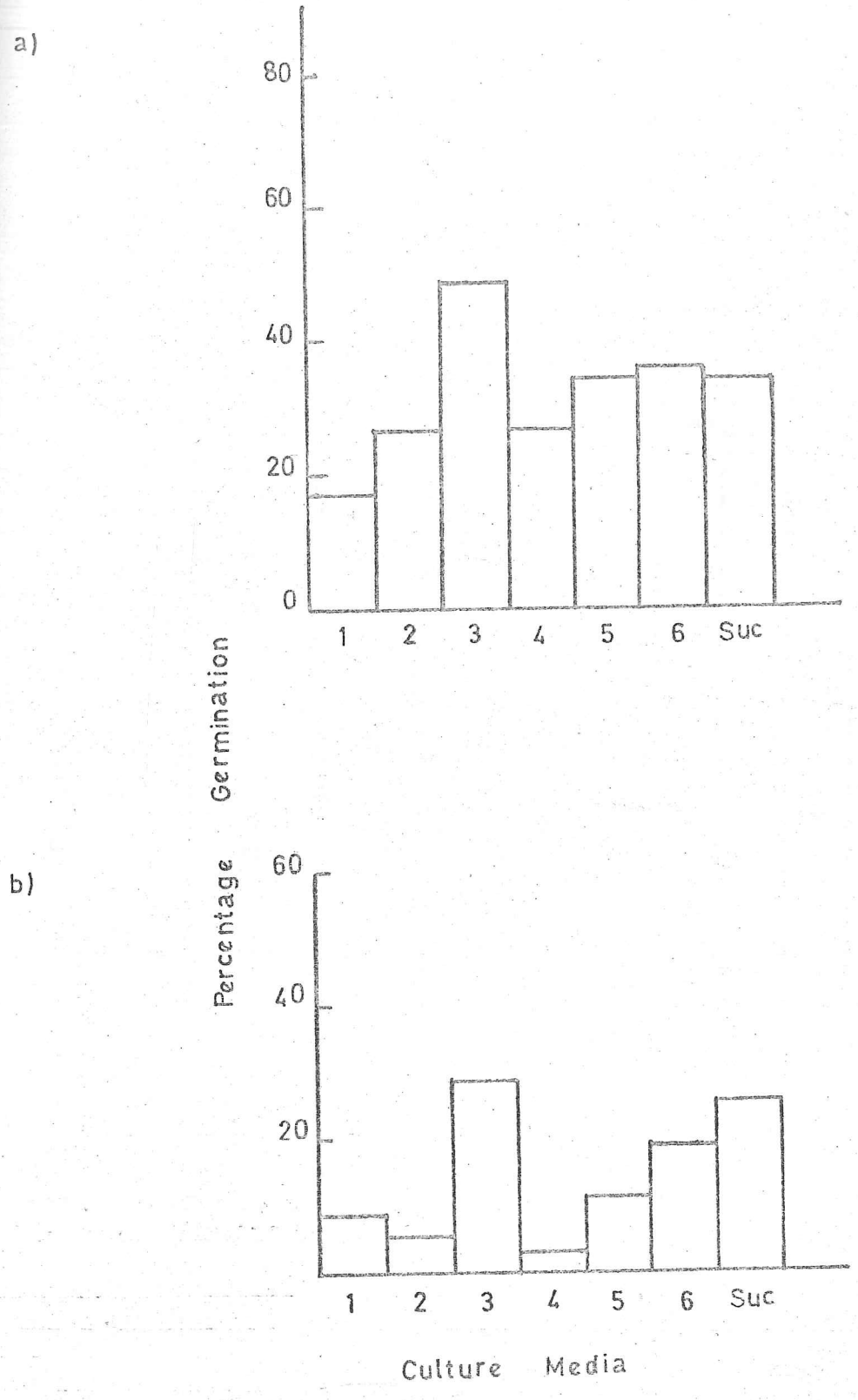


Fig III.4. Average percentage germination on culture media CM1 - CM6 and Sucrose. a) brush inoculation b) spore suspension.
Standard deviations: a = 10.86 b = 10.18

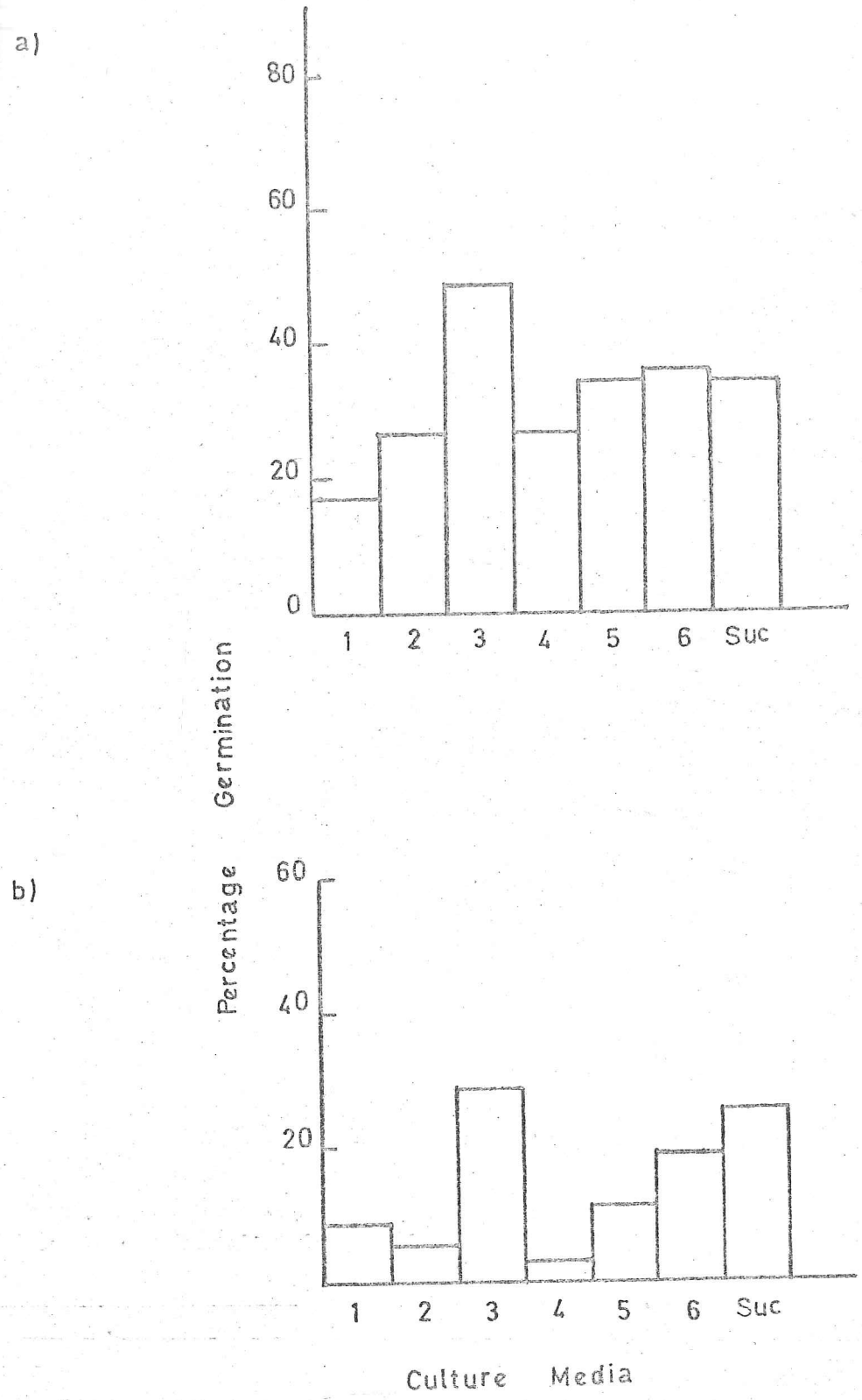


Fig III.4. Average percentage germination on culture media CM1 - CM6 and Sucrose. a) brush inoculation b) spore suspension.
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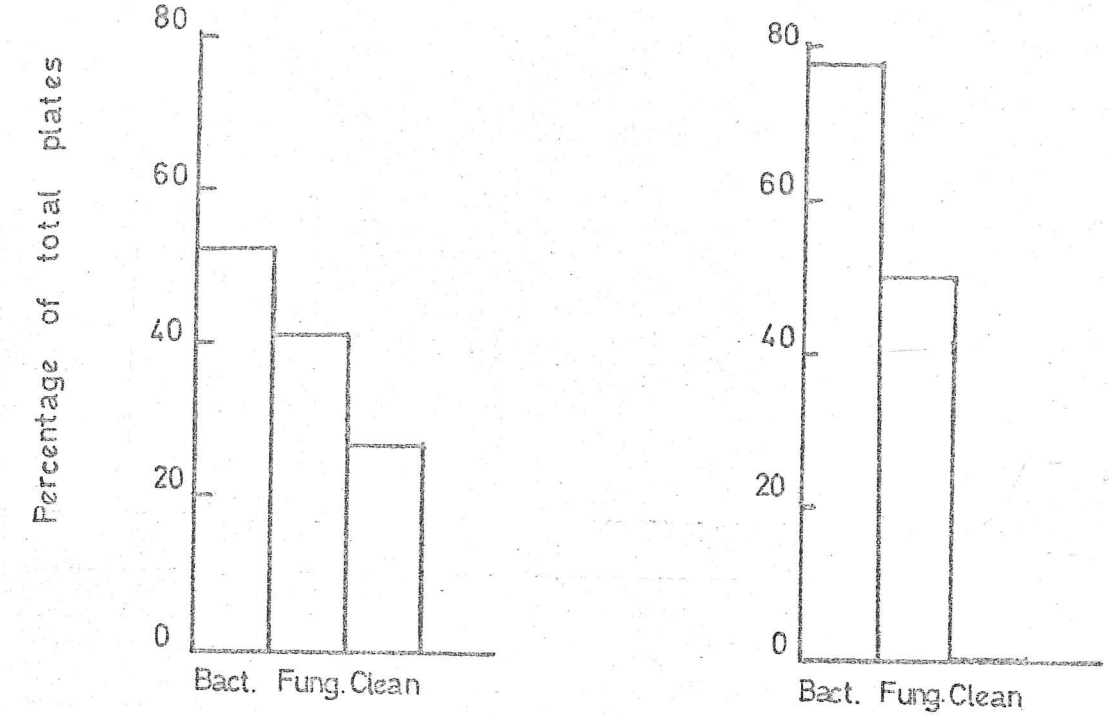


Fig III. 5 Comparative frequencies of plate contaminants. (Totals exceed 100% as some plates had both fungal and bacterial contaminants present.)

Plate III.2a. Swollen germ tube tips and meshwork of fused germ tubes (x 1000).

a

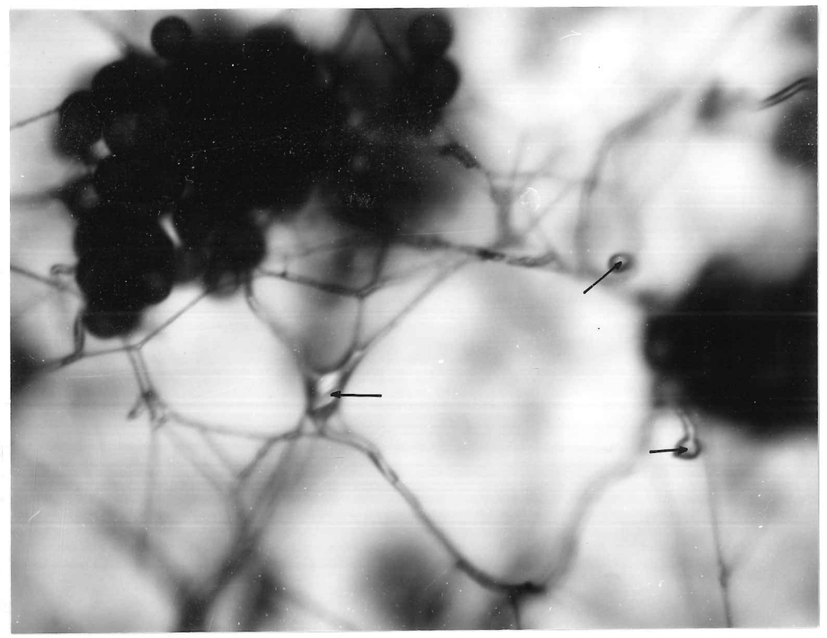
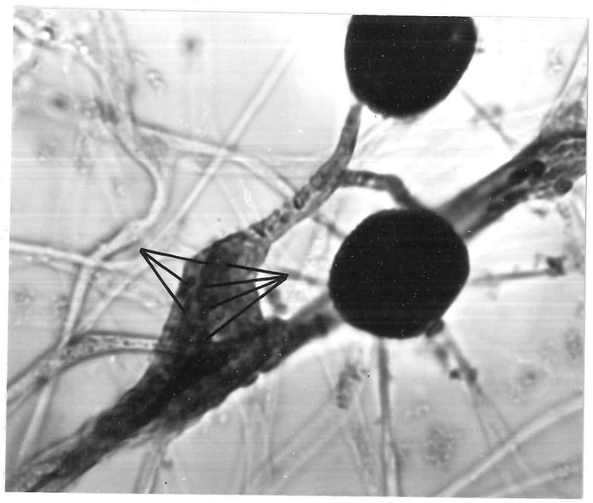


Plate III.2b. Fusion body nuclei (x 1000).

b



3ib. Nuclear size

Electron micrographs showed that young uredospore nuclei contain large dense nucleoli (Plate III.3a). The nucleoli were measured and found to average 1.39 μm x 1.74 μm. The nuclei were also measured and found to average 3.6 μm x 2.5 μm. These measurements were very much larger than those of Little and Manners (1969b) who obtained an average nuclear width of 0.7 μm.

As the larger measurements were obtained from electron micrographs and Little and Manners' observations were from light microscope observations using Flemming's Triple stain, comparative measurements were made using wax embedded sections stained with Flemming's Triple stain, and Mayer's Haemalum with Orange G. Light microscope observations on wax embedded sections stained with Mayer's Haemalum and Orange G gave a darkly stained body within a lighter stained round to oval area (Plate III.4a). Preparations stained with Flemming's Triple stain showed the same darkly stained body, although the lighter surrounding area was frequently less well defined (Plate III.4b).

The measurements of the darker stained bodies correspond with the EM measurements of nucleoli, and those of the lighter area with the EM measurements of nuclei (Table III.5) and therefore are considered to be the spore nuclei and nucleoli.

Table III.5. Comparative measurements of Nuclei and Nucleoli in light microscope and EM preparations

Stains/Preparations	Nucleus		Nucleolus	
	Length	Width μm	Length	Width μm
Flemming's Triple stain	4.59	3.33	1.49	1.28
Mayer's Haemalum & Orange G.	4.06	3.24	1.73	1.29
Electron Microscope	3.57	2.51	1.74	1.39

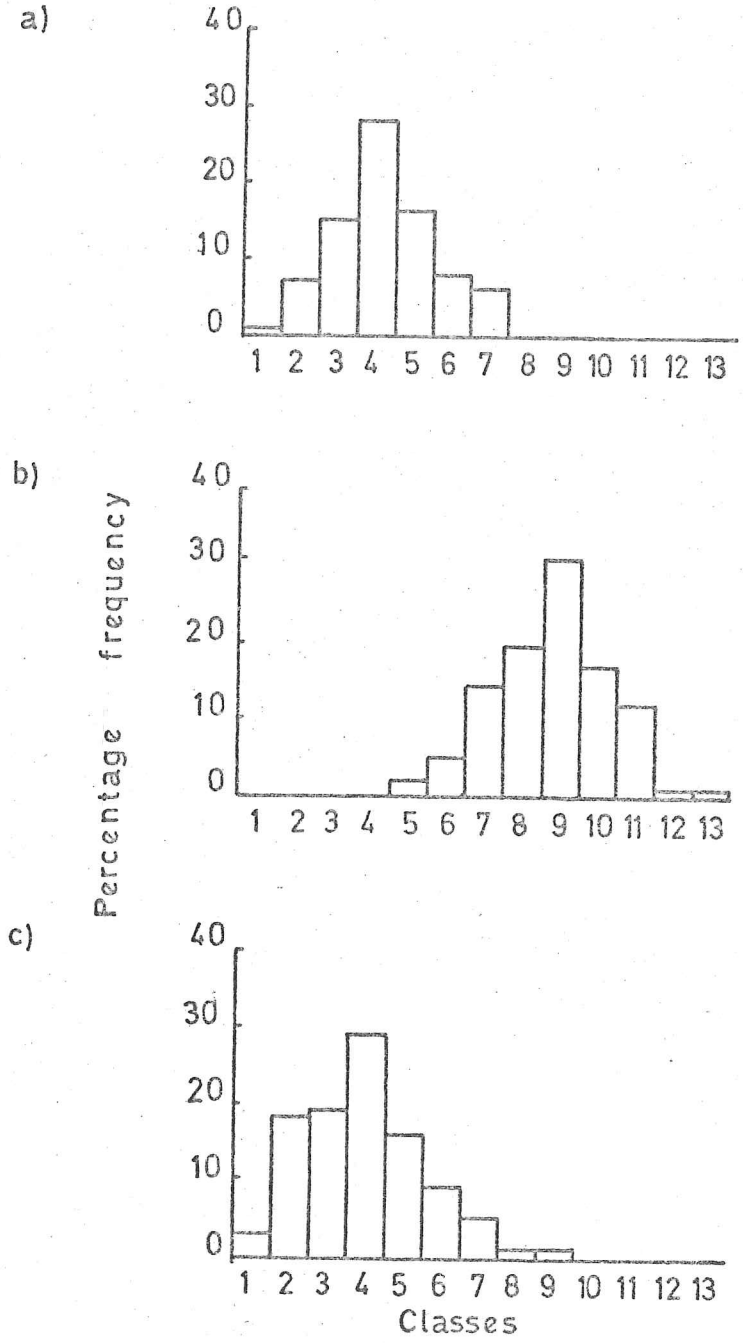
Measurements of germ tube nuclei were comparable with those from spore sections and this meant that germ tube nuclei could be used to compare isolate MB-5 with race 41 E 136 and race 104 E 137. Comparative nuclear sizes are given in Fig. III.6 and nuclei of isolate MB-5 were found to be the same size as those of races 41 E 136 and 104 E 137.

The teleutospore nuclei appeared to be round and not oval, as in the case of the uredospore nuclei, so the nuclei were only measured in one direction, at right angles to the length of the spores.

Forty nuclei were measured from individual spores of the two races, and the average nuclear width was 6.1 μm for race 104 E 137 and 6.2 μm for 41 E 136, approximately twice the dimensions of the uredospore nuclei and from Fig. III.6 the two modes are clearly distinct. All the teleutospores examined were monokaryotic.

3ic. Nuclear number

The uredospores of P. striiformis were generally binucleate, although trinucleate spores were also observed (Plate III.4c). The germ tubes however, frequently contained more than two nuclei, with four and occasionally up to 10 nuclei being present in some. This would indicate that nuclear division had taken place in the germ tubes, as 3 was the maximum number of nuclei observed in the uredospores. Where such nuclear divisions were observed, they were synchronous within pairs (Plates III.5a, c). The nuclear number of isolate MB-5 was also recorded, as the spores from this isolate were significantly larger than the two races 104 E 137 and 41 E 136 (Ch. I.2iii). In all the spores examined, only 2 nuclei were counted.



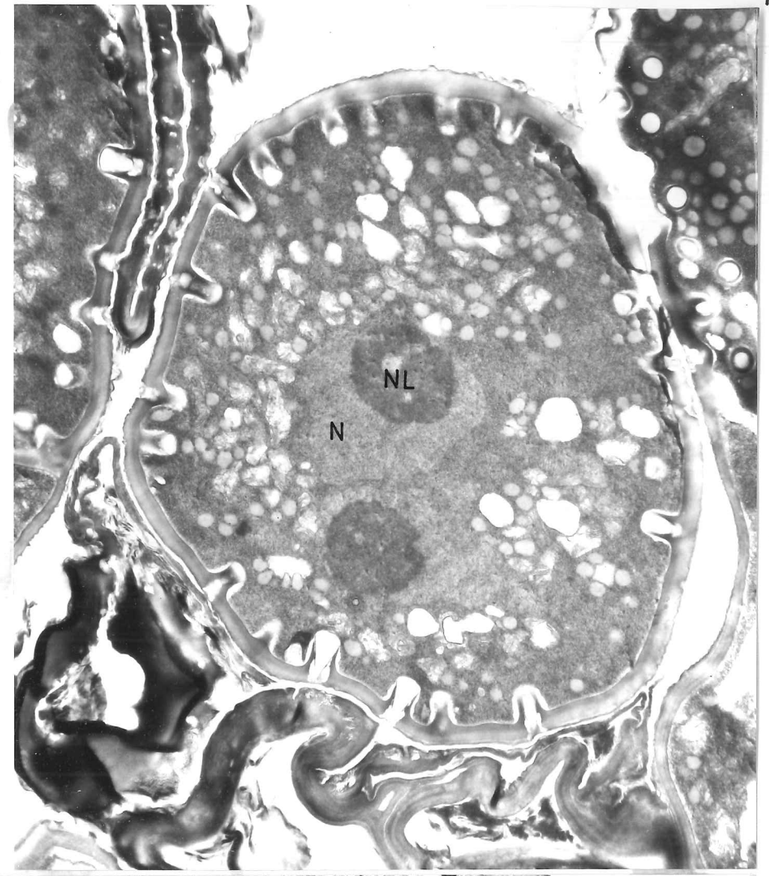
Class.	Nuclear width µm.
1.	2.00 - 2.49
2.	2.50 - 2.99
3.	3.00 - 3.49
4.	3.50 - 3.99
5.	4.00 - 4.49
6.	4.50 - 4.99
7.	5.00 - 5.49
8.	5.50 - 5.99
9.	6.00 - 6.49
10.	6.50 - 6.99
11.	7.00 - 7.49
12.	7.50 - 7.99
13.	8.00 - 8.49

Fig III.6. Relative nuclear sizes of: a) Uredospores, isolate MB-5; b) Teleutospores and c) Uredospores of races 41 E 136 and 104 E 137.

Plate III.3a. Electron micrograph of young uredospore.
N = Nucleus. NL = Nucleolus. (x9000).

Plate III.3b. Electron micrograph of young uredospore,
(Potassium permanganate fixation) (x15,000).

a



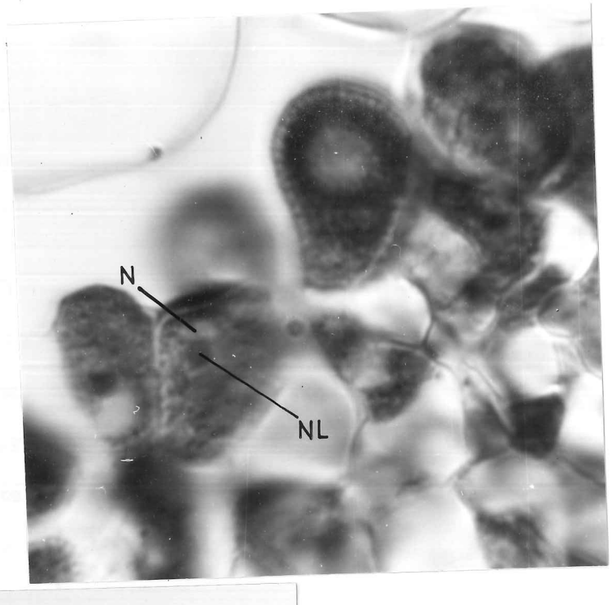
b

Plate III.4a. Section of young uredospores showing nucleus (N) and nucleolus (NL). Stain - Mayer's Haemalum (x 2,250).

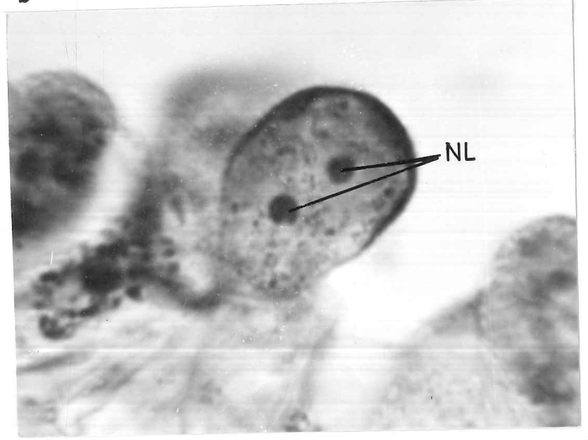
Plate III.4b. Section of young uredospore showing nucleolus (NL). Nucleus is not well defined. Stain - Flemming's Triple (x 2,250).

Plate III.4c. Section of young trinucleate uredospore. Stain - Mayer's Haemalum (x 1,000).

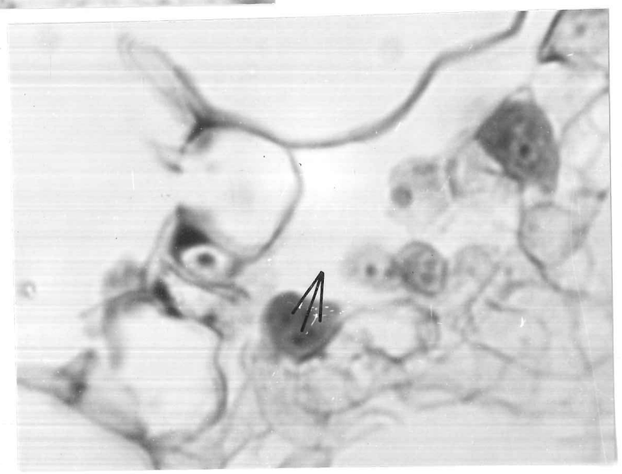
a



b



c



3id. Chromosome Number

Phase contrast and stained preparations were used to assess chromosome number and the preparations were not squashed before examination. This caused some difficulty in photography, but all observations were supported with line drawings.

Considerable difficulty was encountered in making these observations because of the small size of the chromosomes. After staining the preparations with Giemsa-HCl (Chambers, 1970), six deeply stained chromosomes were observed in dividing nuclei at metaphase (Plates III.5b, 5c). In isolated cases late metaphase figures were observed and the chromosomes could be seen to have split longitudinally, but remained joined at the centromere (Plate III.5a). Twelve chromosomes were counted on several occasions. The separation of daughter nuclei was not observed, but the closeness of paired nuclei and the occurrence of 4 nuclei in the germ tubes was evidence that the final stages of the separation had taken place.

3ii. Radioactive Labelling of Spores

3iia. Experiment II

The spores collected from the plants in experiment II, 14 days after inoculation, were placed on 1% water agar containing 0.5% glucose and left overnight at 10°C in the dark. Germination was assessed after 18 hours and is given in Table III.6.

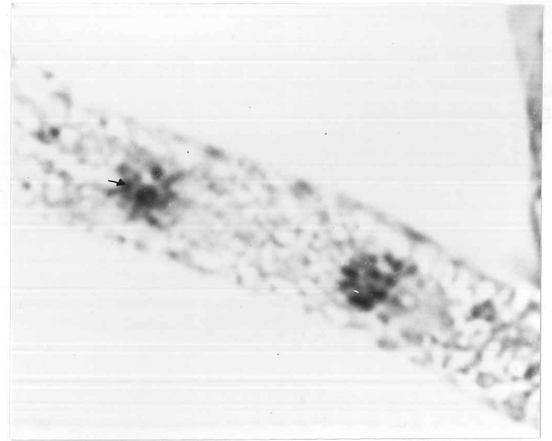
Attempts to transfer the spores from the agar to slides in preparation for autoradiography failed, as the germ tubes frequently broke and became detached from the spores. All later germination was carried out using the 'chamber' technique described above (Ch. III.2ia) and slides were prepared for autoradiography where appropriate.

Plate III.5a. Synchronous division of nuclei. Chromosomes still joined at centromeres. Stain - Giemsa-HCl (x 3,150).

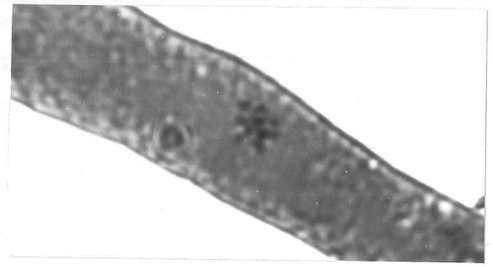
Plate III.5b. Nuclear division. n = 6 (x 3,150).

Plate III.5c. Nuclear division. n = 6.

a



b



c

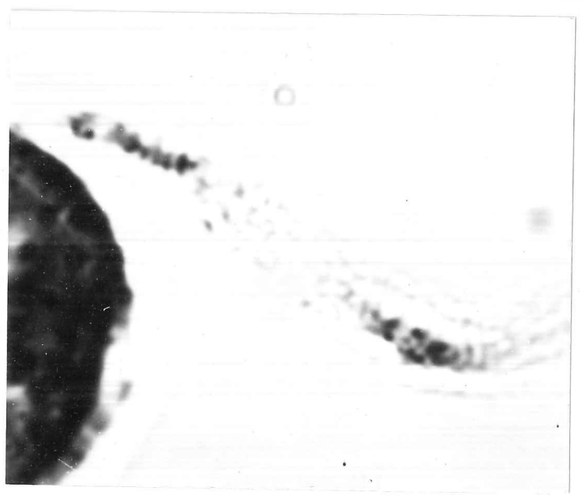


Table III.6. Percentage germination recorded from spores collected from labelled plants 14 days after inoculation

Day on which label applied	Percent Germination*	Comparisons with control
1	30.1	-8.5
2	34.3	-4.3
3	44.0	+5.4
4	35.6	-3.0
5	42.8	+4.2
6	29.5	-9.1
7	13.7	-24.9
8	24.2	-14.4
9-13	No spores	-
Control	38.6	0.0

* Mean of 2 values from each plate.

Although the percentage germination appeared to be little affected by the time at which the label was applied, the distribution of incorporated thymidine through the plant varied considerably (Fig. III.7). Unfortunately most of the samples from day 11 were lost during treatment, but from the time flecks became observable on the leaf up to the time of sporulation, there was a marked increase in the uptake of label. Following the onset of sporulation a rapid decrease in uptake by the first leaf was observed and there was evidence of a slight increase in uptake in the 2nd and 3rd leaves.

The result of this experiment showed that the maximum uptake of ^{3}H-Thymidine by the first leaf occurred during the period between flecking and sporulation. This coincides with rapid mycelial spread and spore development in the rust, so it can be suggested that the bulk of the uptake at this time is into the pathogen.

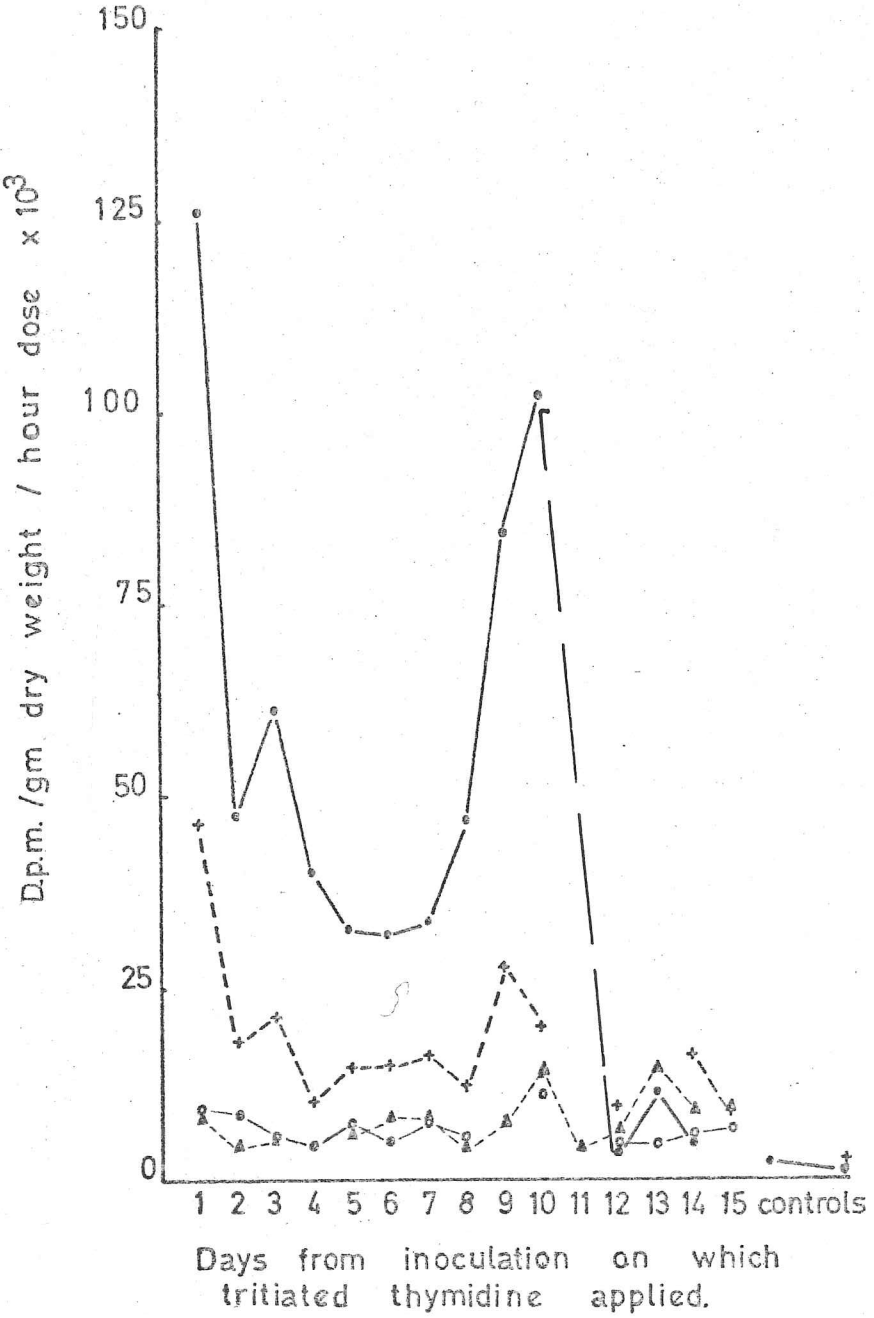


Fig III.7. Distribution of incorporated ^{3}H- thymidine in leaves of wheat seedlings, 18 days after inoculation of the first leaf with race 104 E 137 of P. striiformis.
•——• 1st leaf +——+ 2nd leaf ∘——∘ 3rd leaf
▲——▲ 4th leaf

3iib. Experiment IV

The condition of the spores obtained from the three collection methods probably had a considerable influence on their viability.

The spores obtained from method II were sometimes stuck to the bottom of the tube by condensation, which occurred due to the restriction of air flow. In method III however, although there was a ring of condensation around the rim of the tube, the spores were dry at the bottom of the tube. The spores from method I were also dry.

The germination tests using the glass chamber technique described above, indicated that both methods II and III gave better germination than did method I (Table III.7) Method III was adopted for further work, as the spores were collected dry, and showed the best germination levels.

Table III.7. Percentage germination obtained using three different collection methods.

Method 1 percent germination	Method 2 percent germination	Method 3 percent germination
3.9	47.1	78.8
6.0	33.9	50.1
5.4	53.9	88.4
3.7	56.4	85.5
$\bar{x}$ 4.8	47.8	75.7

$P = <0.001$

3iic. Experiment V

Spores were collected from each set of six leaves, 6 and 12 days from the first application of ^{3}H-Thymidine. The germination chambers were prepared and the spores derived from labelling treatments and spores from control (unlabelled) treatments were placed on distilled water in the following combinations:

	Labelled	Unlabelled
1	104 E 137	41 E 136
2	41 E 136	104 E 137
3	104 E 137 and 41 E 136	-
4	104 E 137	-
5	41 E 136	-
6	-	104 E 137
7	-	41 E 136
8	-	104 E 137 and 41 E 136

The chambers were left for 24 hours at 10°C in the dark, to allow for maximum opportunity for fusions of germ tubes to occur.

Germination of the labelled and unlabelled spores was assessed for both races individually (Table III.8) and for the mixed race combinations.

Lower levels of germination were obtained from the spores with a time lapse of 1-5 days than 7-10 days. The lowest values were obtained with 3-4 days lapse in race 104 E 137, but there was more variation for race 41 E 136.

The developed autoradiographs were examined and 40 stained nuclei were measured and the number of darkened grains over each nucleus were counted (Plate III.6a). A similar total area of background was also examined and the number of darkened grains within this area was also

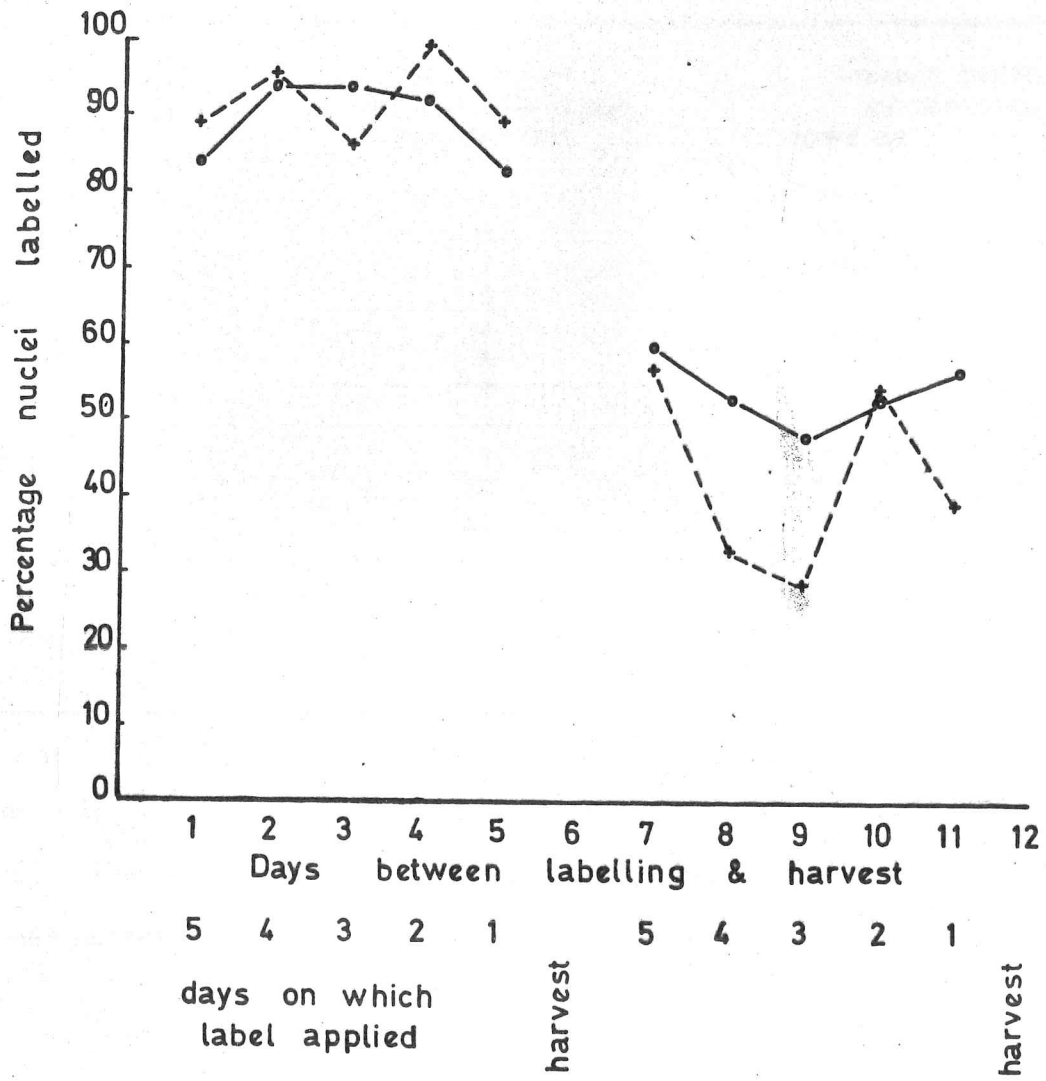


Fig III.8. Relationship between % nuclei labelled and time lapsed between the application of ^{3}H-thymidine to the plants and harvest.
+ race 41 E136 • race 104 E 137

Table III.8. Germination recorded from labelled spores of race 41 E 136 and race 104 E 137 of P. striiformis

Day on which label applied	Days lapsed between labelling and harvest	Average percent germination 104 E 137	41 E 136
Control		37.1	44.5
5	1	27.9	26.7
4	2	30.4	39.1
3	3	19.1	31.4
2	4	20.4	44.1
1	5	31.3	39.5
5	7	43.8	31.3
4	8	41.5	45.5
3	9	47.9	43.1
2	10	54.8	38.3
1	11	37.5	44.8
Control		40.1	57.8

Means of 2 values

examined; the numbers of darkened grains per 10 μm^2 were calculated. Despite the relatively high level of background, there was a significantly higher number of darkened grains over the nuclei:

Average number counted per 10 μm^2 background = 18.3

Average number counted per 10 μm^2 nucleus = 31.1

L.S.D. at 5% = 4.50.

All slides on which only one labelled race had been germinated were systematically scanned and 250 nuclei per slide were recorded for the presence or absence of ^3H. The percentage labelled nuclei for each day was plotted (Fig. III.8) to determine the required time between the application of label and spore collection to achieve the maximum percentage of labelled spores. The results were also compared with percent germination.

As thymidine incorporation can only take place during DNA synthesis, it would appear that the maximum rate of incorporation takes place 2-4

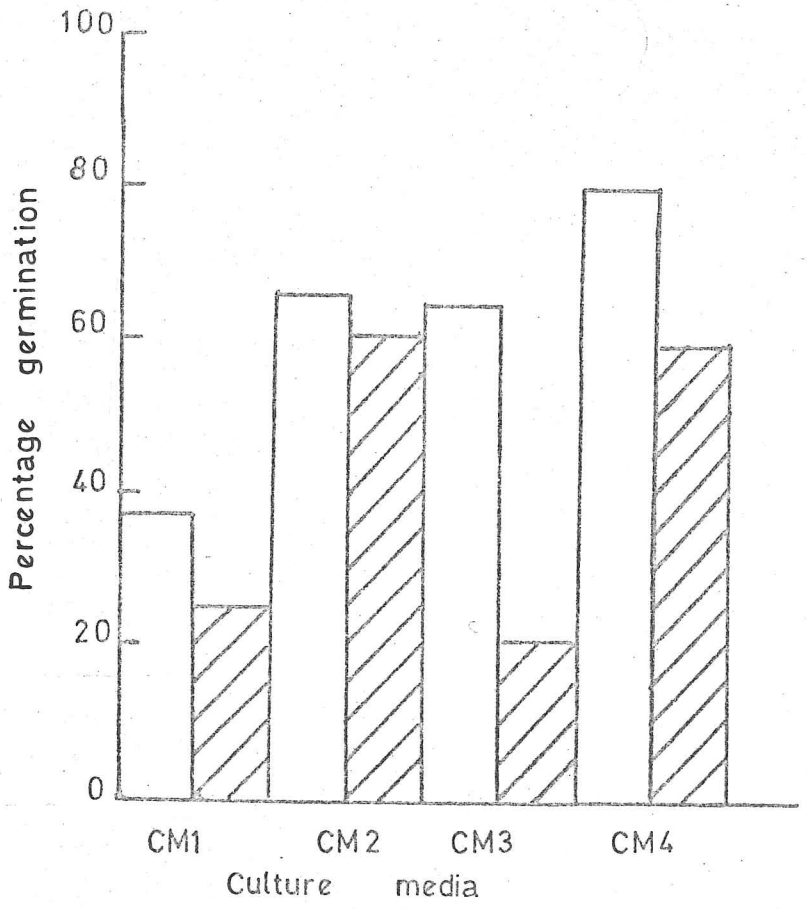


Fig III. 9. Comparative germination at ▨ 7°C in dark ▭ 10°C lit

days after flecking, and that label applied between flecking and sporulation should be readily incorporated, thus providing a race marker.

The slides on which labelled and unlabelled spores had been germinated were then systematically scanned to detect germ tubes in which labelled and unlabelled nuclei were present, but the empty germ tubes were not easy to detect through the film, and many had been broken so it was not always possible to follow the entire length of the germ tube, though seven germ tubes were observed with both labelled and unlabelled nuclei present.

3iii. Axenic culture

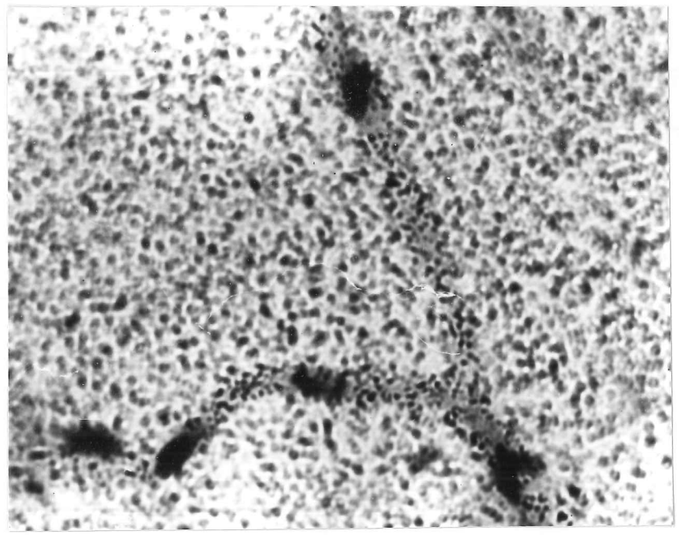
On the media detailed in Table III.1, consistently higher percentage germination was obtained at 10°C with illumination than at 7°C in the dark (Fig. III.9). The germ tubes produced at 10°C were elongated and without cross walls or branching. At 7°C, although the percentage germination was lower than at 10°C, the germ tubes were shorter and mostly showed signs of branching after 4 days incubation.

Four days after the sets of plates were seeded, most of the germ tubes on the CM1-CM4 plates had burst, discharging their contents onto the medium (Plate III.6b). The intact germ tubes had given rise to short branches. Some sporelings on the sucrose and CM6 plates developed structures similar to substomatal vesicles, which in turn produced one or two infection-type hyphae (Plate III.7a). The infection-type hyphae were only observed arising from these vesicle-like bodies, and differed considerably from the branched hyphae which did not arise from the vesicles. In some cases further branching was observed (Plates III.7b, 7c). Growth was also observed to occur under the surface of the medium.

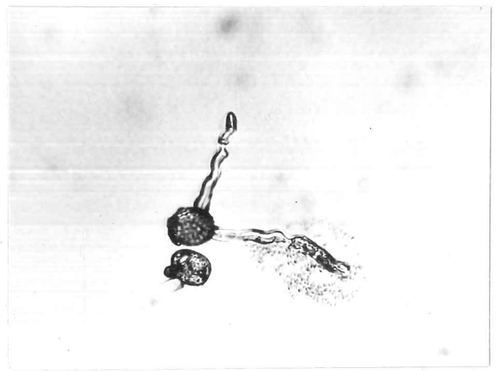
Plate III.6a. Autoradiograph showing labelled nuclei. (x 1000).

Plate III.6b. Germ tube which has discharged contents onto the agar.

a



b



After 7 days, most of the germ tubes on the CM1-CM5 plates had burst, and those which had not, had shown little development from earlier examination. On the sucrose plates and CM6 however, some further growth had taken place, and there were more germ tubes growing into the agar. By 14 days, the sporelings on the sucrose agar showed no signs of staling, though there was no evidence of continued growth.

Germination was comparable in sealed and unsealed plates, although burst germ tubes were more frequent in sealed plates, and plates were left unsealed in further work. It was found that the presence of bovine serum in the media reduced the number of burst germ tubes.

Germination was poor (maximum 23%) on the test media II after 24 hours at 7°C, although the best germination (15% or more) was observed on the media indicated *** in Table III.2. After a further period of 24 hours in an illuminated cabinet, germination was greatly increased on all media, to over 90% on media indicated *** in Table III.2.

Vesicle-like bodies were again observed giving rise to fine infection-type hyphae. The period of active growth ranged from 6-8 days on the media shown * in Table III.2, through 8-10 days **, to 10-13 days ***. On media marked ***, considerable branching and some germ tube fusions were observed. This would suggest that 1.0-0.5% peptone enhanced growth, but that the concentration of yeast extract and the presence or absence of serum were less important factors. The media giving relatively poor initial germination gave rise to better growth.

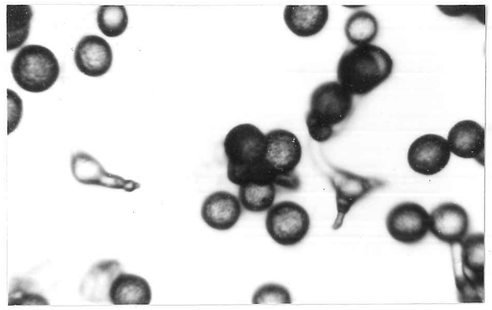
The media containing Simmons Citrate agar (Table III.3) as the only mineral supply supported little growth. The addition of Czapek minerals to these media improved germination and enabled growth to be maintained for 13 days on media 1c+, 2c+, 4c+, 5c+ and 6c+. There was considerably

Plate III.7a. Vesicle-like bodies giving rise to one and two infection-type hyphae (x 1000).

Plates III.7b & 7c. Sporelings of P. striiformis showing further branching (x 250).

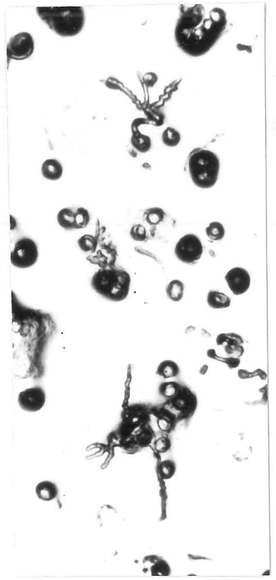
Plate III.7d. Yeast-like budding. (x 1000).

a



b

c



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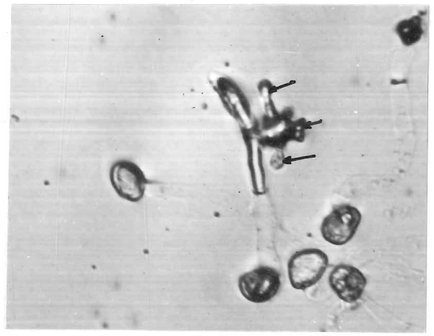

less growth on media lacking bovine serum than on those media containing the serum, in contrast to the results from the second set of media, in which the presence or absence of serum had little noticeable effect on growth. This would suggest that the Simmons Citrate agar was either insufficient for, or contained some inhibitor of the growth of P. striiformis, and that the serum either supplemented the nutrient supply or neutralised the effect of the inhibitor.

The exudates from senescing leaves improved the continued growth of the pathogen on the single source media, despite relatively poor germination observed on some media (Table III.4). Casamino acids, Peptones and Yeast Extract gave good germination, but growth was not maintained after 8 days. Czapek minerals and Malt agar both supported growth for 15 days despite poor germination levels: in one case on Czapek minerals a sporeling developed lateral branches and a sporelike structure at the tip of one branch (Plate III.7d). Spores on water agar continued to grow for 13 days, presumably relying on leaf exudates. The spores on Simmons Citrate agar alone failed to germinate, again suggesting the presence of an inhibitor.

III4. Discussion

4i. Cytological observations

The minimum dimensions of the nuclei obtained from this work differ considerably from Little and Manners' (1969b) observations of 0.7 μm, but they were consistently obtained, and are supported by electron microscope observations and measurements.

Fleming's Triple stain uses crystal violet as the nuclear stain, which requires differentiation in clove oil. The resistance of the spore wall slows down the staining and destaining process, and after sufficient

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Fleming's Triple stain uses crystal violet as the nuclear stain, which requires differentiation in clove oil. The resistance of the spore wall slows down the staining and destaining process, and after sufficient

stain from the spore wall and cytoplasm, only a small darkly stained object remained. Initially this was assumed to be the nucleus. However, when sections stained with Mayer's Haemalum showed similar deeply stained bodies, surrounded by a distinct lighter zone, other alternatives were considered.

Electron microscope preparations showed a large electron dense nucleolus to be present in the young uredospores, with the surrounding nucleus being less dense, and surrounded by a membrane. Preparations fixed with potassium permanganate dissolved the nuclear material leaving the double nuclear membrane surrounded by the spore cytoplasm (Plate III.3b). The width of the areas surrounded by the membrane were comparable to the measurements of the nuclei in preparations fixed with gluteraldehyde, and greatly exceeded the dimensions of the nucleoli.

The size of the nucleolus varies with the age and development of the spore and is large in young spores but decreases in size as the spores mature (Dunkle et al., 1970; Mitchell and Shaw, 1959). This would account for some of the variation in the size of the nucleolus within this work, and when compared to Little and Manners' observations.

Attempts to germinate teleutospores in order to obtain chromosome counts did not succeed, but the measurements of the teleutospore nuclei were approximately twice the size of uredospore nuclei.

Although isolate MB-5 had significantly larger spores than did races 41 E 136 and 104 E 137, its uredospore nuclei were similar in size to those of the two races.

The infrequency with which more than two nuclei per spore were observed illustrates the general stability of nuclear number within P. striiformis. Little and Manners found that the uredospores of

isolates SSC-4 and SSC-21, which contained the partly combined virulence of races 8B (32 E 128) and 2B (40 E 72), frequently had 3 nuclei per spore, which would suggest potentially unstable isolates as in the case of the trinucleate Khapli-infecting biotype of P. graminis (Nelson et al., 1955). However, cultures SSC-4 and SSC-21 remained stable throughout their work, unlike the Khapli infecting biotype which gradually dissociated into binucleate cultures. In this work isolate MB-5 (race 105 E 137) which combines the virulence of races 41 E 136 and 104 E 137 was seen to be binucleate similar to the 'parental' races which are common races of P. striiformis. This consistency in nuclear number could be taken as evidence that the new race 105 E 137 was a stable race, which could readily become established if introduced to field conditions.

The small size of the chromosomes in the rust fungi has deterred investigations of their chromosome numbers and behaviour. Mc Ginnis (1953) reported the length of chromosomes of P. graminis to range from 0.8-1.4 μm in length, and 0.3-0.5 μm in width. Recent work by Valkoun and Bartos (1972) showed that the chromosomes of P. recondita were 0.5-1.2 μm in length.

The chromosomes of P. striiformis also fit into a similar size range, and are approximately 0.7 to 1.1 μm in length. Six chromosomes of these dimensions would not easily be accommodated within a nucleus of diameter 0.7 μm.

Three, four and six chromosomes have been reported among the rusts (Mc Ginnis, 1953, 1954, 1956; Valkoun and Bartos, 1972) and Mc Ginnis has suggested that the basic chromosome number 4 is typical of homothallic and 3 or 6 is typical of heterothallic species.

Six chromosomes were observed in this work and 12 were observed on several occasions. At no stage were 4 chromosomes recorded. This would mean that if Mc Ginnis' hypothesis is correct, P. striiformis is heterothallic as was suggested by Little and Manners (1969a).

Germ tube fusions and cytoplasmic streaming were frequently observed and have been reported in the past (Little and Manners, 1969b). Little and Manners suggested that nuclear exchange could take place as a result of this, although nuclei were not seen to move from one germ tube to another.

Wilcoxson et al. (1958) observed nuclei in fusion bodies of P. graminis. The discovery of large numbers of nuclei, up to seven observed, within the fusion bodies in P. striiformis is therefore of considerable interest, as it is evidence that the nuclei may leave the germ tubes in which they originate, and in this situation nuclear reassortment could take place.

It has been observed that the nuclei divide synchronously in pairs, and that the pairs of dividing nuclei studied have been in close proximity throughout the division. This would mean that in the event of any malfunction during the course of division, chromosomal exchange may be able to take place.

4ii. Radioactive labelling of P. striiformis

In the pilot experiment designed to detect any ^{3}H-Thymidine uptake by the spores, no significant differences in the number of counts were observed between the spore samples and the blank controls, however, as few spores could be collected, a very small sample of spores was used in each case, so any ^{3}H-Thymidine present would have been in very small concentrations.

One of the main problems was to obtain sufficient labelled spores to produce a significant number of scintillations to be measured by the techniques of liquid scintillation, and to retain enough spores to use for autoradiography. For this reason, the distribution of label in whole infected and uninfected leaves of the same plants was studied using the liquid scintillation counter. It can be argued that, if mycelium was developing rapidly in a leaf, then a large quantity of label entering that leaf would probably be incorporated in the pathogen, especially if the leaf was already fully expanded. In the results of experiment II, it was observed that much label entered leaf 1 in the period between the occurrence of visible flecks and the commencement of sporulation on the leaves.

A high rate of uptake was also observed into leaf 1 on the first day after labelling (**Fig. III.7**). This may have been caused by conditions in the glasshouse, when the seedlings were transplanted to the thimbles in culture solution. The temperature in the glasshouse reached 43°C as the result of an electrical fault.

As the seedling leaf was inoculated when it was fully expanded, one would expect very little uptake in this leaf, and the bulk of the thymidine to go to the developing 2nd, 3rd and 4th leaves. The first leaf, however, showed the largest amount of incorporated thymidine present until day 12 and showed a sharp increase between fleck and sporulation. This is the period during which the most rapid development of the pathogen takes place. The uptake of ^{3}H in the fully expanded first leaf is therefore comparable to the stage of development of the pathogen.

Spore production lasted for a relatively short period of time in this experiment, 5 days, and this was probably due to the heat (43°C) and mechanical damage to the plants, through handling during the transfer to and from the labelled solution. An optimum time lapse of 2-3 days

between applying the label and harvesting the labelled spores was observed in experiment V, and this established that 2-3 days was the time taken from spore initiation to spore release. It would seem likely therefore that only the early developed pustules gave rise to sporulation in experiment II, and this would account for the lack of ^{3}H-Thymidine uptake by the first leaf after the onset of sporulation. In experiment V, however, thymidine incorporation continued to take place after the onset of sporulation, but spore production also lasted longer, 9 days. As spore development had not been halted, thymidine incorporation was able to continue in developing spores while sporulation was taking place.

The condition of the spores produced had a considerable effect on the germination obtained, as can be seen from experiment IV. The spores collected using method I were comparable to those collected by the normal cyclone method (Tervet and Cassel, 1951), and had been produced in an atmosphere of relatively high temperatures and low R.H. The spores collected in specimen tubes in experiment IV were produced in a humid atmosphere. These spores gave better levels of germination than the other method, and it is possible that the high humidity during spore production improves germination. It has been observed that spores produced on the isolation bench exhibit a higher level of germination than those produced in glasshouse compartments. The hydration of uredospores before inoculation has been shown to improve germination (Mc Cracken and Burleigh, 1962; Strobel, 1965; Tollenaar and Houston, 1965) and it is possible that if the spores mature in a high relative humidity, the effect of the germination inhibitor may be reduced in a similar way.

The spore collection method chosen, method III gave the highest percent germination, and also reduced the possibility of spore loss and radioactive contamination of the glasshouse.

In experiment II, no spores were produced by the plants to which label was applied after 8 days from inoculation. The germination of the spores produced from plants labelled before the 9th day showed that viability was decreasing on days 7 and 8, which corresponds with the increasing uptake of ^{3}H to the first leaf during this period. In experiment V, the label was applied during this period, and race 104 E 137 showed a negative correlation between percent nuclei labelled and percent germination. It would therefore seem likely that the presence of the radioactive label during this time of rapid nuclear division may be having a detrimental effect on spore viability and possibly, on spore development.

It is interesting to notice that race 41 E 136 appears not to show this correlation between labelled nuclei and percentage germination. This could suggest a higher degree of tolerance to the effects of β radiation, as the variation in germination observed is typical of germination from similar unlabelled spore samples.

It was not considered necessary to illustrate biochemically that the ^{3}H-Thymidine was present in the fungal DNA, as the autoradiographs illustrated that the ^{3}H was associated with the nuclei, and the ^{3}H was only being used as a tool to mark nuclei for identification, not to study thymidine incorporation into fungal DNA.

Unfortunately it has not been possible to illustrate with any certainty whether nuclear exchange has taken place during the germination period. Many fusions have been observed, but the germ tubes were too long to allow convenient observation, and multiple fusions involving several germ tubes prevented explanation of nuclear movements. Shorter periods of time for germination could reduce these problems, as this should give shorter germ tubes and fewer fusions.

As Giemsa-HCl is a nuclear stain, it did not stain cytoplasm or germ tube walls, which left considerable lengths of germ tube with little stainable material present. This meant that most of the germ tubes were extremely difficult to follow through the photographic emulsion, and it was often difficult to trace germ tube nuclei to their parental spores. Attempts to counter stain germ tube walls with lactophenol cotton blue were not satisfactory: the stain destained the nuclei as Giemsa-HCl is pH dependent for its differentiation, and the lactophenol dissolved the emulsion.

Feulgen stain has been used successfully with rust fungi (Williams and Hartley, 1971), but it has not been used in this work.

It is possible that germ tube fusions during germination are not important in the development of races, but that fusions of mycelium within the leaf provide opportunities for nuclear exchange.

The work described here has shown that it is possible to label nuclei of P. striiformis and to observe labelled nuclei in the germ tubes of germinating spores. The development of the autoradiographs took 6 months on each occasion, and even after this period of time the amount of radiation detected was not easily distinguishable, visually, from background effects. It was therefore difficult to perform experiments with modifications based upon the results of earlier experiments. In the cases reported here difficulties were encountered in observing the relationships between germ tubes, and the nuclei they contained. These factors and conflicting theories (Taylor, 1958; Woods and Schairer, 1959), regarding the transmission of label during nuclear division, meant that it was not possible to interpret the presence of labelled and unlabelled nuclei in close proximity in the same germ tube as being indicative of nuclear exchange between different races of the

pathogen. However, further development of these techniques might be possible which would help to establish the occurrence and relative importance of heterokaryosis as a source of variation in P. striiformis.

4iii. Axenic culture

The maximum growth obtained was on malt agar, CM6 Sucrose and Czapek minerals. In these cases, vesicles were formed which gave rise to infection type hyphae, some of which gave rise to further branching. In one case there was evidence of yeast-like budding and the formation of a spore-like structure. Williams (pers. comm.) found this phenomenon to be common when he attempted to grow P. striiformis hordei in axenic culture. He suggested that this budding indicated the first stages in achieving axenic culture of the pathogen.

Vesicle-like bodies were observed in all three experiments, giving rise to infection-type hyphae. Manners (1950) and Straib (1940) also observed similar bodies and observed that they could be homologous with substomatal vesicles. This evidence would support Chakravorti's observation (1966) with P. graminis that appressorial and vesicle formation is not dependent on a cuticular membrane. Only germ tubes which had formed these vesicle-like bodies gave rise to any growth comparable with sporelings of P. graminis (Williams et al., 1966, 1967). This work would also suggest that in P. striiformis vesicle formation is essential for the transformation of a germ tube to an infection-type hypha on artificial media, although in the leaves (Ch. II.3iii) vesicles are not always formed.

Germ tubes were seen to grow under the surface of the media. This has not been reported in other rust fungi, but would be advantageous if the 'leakiness' of the hyphae permitted absorption of nutrients through

the hyphal walls. There was no evidence of haustoria-like bodies being formed, though the germ tube tips were sometimes slightly swollen.

The incubation conditions affected both germination level and hyphal growth. This could have been due either to the light or to temperature. Tollenaar and Houston (1966a) showed that light promoted germination at 11°C, and Mc Cracken and Burleigh (1962) suggested that germination is not light sensitive at low temperatures, but becomes so in the 10-15°C range. In this work the results show that germination was better at 10°C with lights than at 7°C in the dark, though short branched germ tubes were obtained in the dark. Scott (pers. comm.) has found that germination was not a good criterion on which to assess the potential of a medium for culturing the rusts. He found that long unbranched germ tubes did not give rise to colonies in P. graminis, whereas the short branching germ tubes with cross walls frequently did. The phenomenon of delayed germination has also been observed by Williams (pers. comm.) and Maclean (1971). Williams suggested that it was possible that spores which germinate later give rise to shorter branching germ tubes, which are more likely to give rise to the successful growth of the rust fungi.

It was difficult to correlate germination with the nutrient supply, although the presence of Simmons Citrate agar always reduced the level of germination. The uredospores of P. striiformis will germinate on most media from water to complex nutrient agar. No germination was observed, however, on Simmons Citrate agar alone, and the level of germination obtained on culture media containing Simmons Citrate agar was noticeably less than on other media.

It was evident that 2% sucrose was necessary to establish growth, as Czapek minerals without high levels of sucrose or glucose did not appear to benefit the sporelings. Of the peptone sources, CM6 contained peptonised milk and peptone P which could have aided growth.

In the second set of media the amounts of sucrose and Czapek minerals were uniform throughout, 2% and 4.5% respectively. Treatments 5 and 6 were better than 1-4 and had a higher level of yeast extract. With 0.1% peptone present and 1.0% yeast extract, there was good development. This would suggest that if the peptone concentration is at the 0.5 to 1.0% level, the influence of the yeast extract is masked, but at 0.1% peptone, a higher level, 1.0% yeast extract supplies sufficient nutrients to compensate for the lower peptone level.

The presence or absence of bovine serum had no consistent effect on the growth of sporelings in experiment II, as optimal development was obtained on media with and without the serum. In experiment III however, where Simmons Citrate agar was the only mineral supply, only three of the media, all of which contained the serum, supported any growth. When Czapek minerals were added to the Simmons Citrate agar, the serum was again necessary to support growth. This would suggest that Simmons Citrate agar was either insufficient for, or contained some inhibitor of the growth of P. striiformis.

Simmons Citrate agar differs from Czapek minerals by the presence of citrate and bromothymol blue, and the absence of nitrate and ferric ions. Any of these factors might account for the difference between the two media. One possibility is the absence of ferric ions from Simmons Citrate. The ferric ion content of the serum would then alleviate the deficiency caused by the lack of iron. The pH indicator bromothymol blue might also

be an inhibitor of germination, and the serum, in some way, reduces the inhibitory effect of the dye.

Malt agar is unable to support growth in P. graminis (Williams and Maclean, pers. comm.) and yet has given good results with P. striiformis, and vesicles and infection-type hyphae were often observed.

Germ tubes which had burst and discharged their contents on to the medium were often observed. This was most frequent on CM1-CM5, all of which had relatively low sugar concentrations. It is possible that the osmotic pressure of the medium was less than in the germ tubes, thus creating an osmotic gradient, which could have caused the absorption of water into the hyphae, eventually causing them to burst. Bursting was still observed on media containing 2% sucrose, but burst germ tubes were more frequent in the plates which had been sealed to reduce the possibility of contamination being picked up during the handling. Macko and Fuchs (1970) found that when germinating spores of P. striiformis were exposed to 5% carbon dioxide, swelling of the germ tube tips resulted: at concentrations greater than this, the contents were extruded. The carbon dioxide concentration would have been greater in the sealed plates than in the unsealed plates and could have been a possible cause of bursting. Morgan (1963) observed that Bacillus pumilis was able to lyse spores and germ tubes of rust fungi, causing the cell walls to disintegrate and release the contents onto the leaf or agar surface. Although there was no noticeable correlation between bacterial contamination and bursting, the bacteria were not identified from the plates, so this remains a possible explanation for the observed bursting. The addition of bovine serum reduced the amount of bursting, though it was not evident whether leakiness was reduced.

Considerable difficulty was encountered in distinguishing between live and dead cultures. All turgid hyphae with visible cell contents were considered to be living; cultures were considered dead when a brown substance was seen to have diffused into the medium and the hyphae appeared partly collapsed and empty.

Brush inoculations reduced contamination when compared with the spore suspension method. This would suggest that the bacteria and any fungal spores on the leaf surface were dislodged by shaking the distilled water to suspend the spores, while not so many were transferred by the brush technique.

Insufficient growth was obtained from any of the cultures to provide mycelium free of host tissue for cytological observations. Cytological studies would have been possible on the limited growth obtained, but the cultures were not used as they were left to allow maximum growth to take place and the plates were not discarded until the cultures were showing signs of staling.

To summarise, therefore, nutrients which have been shown to aid growth, and those which have been shown to restrict and/or not aid growth are outlined in Table III.9.

Table III.9. Summary of nutrients tested and suitability for use in the culture of P. striiformis

Media shown to enhance growth		Media showing no beneficial effect & limiting growth*	
Bacteriological peptone	1%	Bovine serum, except in presence of Simmons Citrate agar, when can enhance growth.	
Bacto peptone	1%	Casamino acids	1%
Bovine serum		Evans peptones	0.1%
Czapek minerals	4.5%	Peptonised milk	1%
Evans peptones	0.5-1%	Simmons Citrate agar*	2.3%
Leaf exudates		Sucrose	>2%
M.R.V.P.	1%	Yeast extract	1%
Malt agar	2%		
Peptone P	1%		
Sucrose	2%		
Yeast extract 1% if peptones present	0.1%		

IV. CONCLUSIONS

The production of the new race 105 E 137 from controlled experiments in spore-proof glasshouses illustrates one of the ways in which variation in P. striiformis can arise. The combined virulence of the two races being used has given rise to a third race with the ability to infect the host varieties susceptible to either one of the races. This phenomenon has not been confined to glasshouse experiments, as a race provisionally identified as race 45 E 140 contains the additive virulence of two races 37 E 132 and 41 E 136.

The occurrence of these two isolates must be considered in the light of field tests on new wheat varieties, and some concern is aroused by the practice of using mixed race infections in field conditions, although such tests are necessary to establish whether new varieties contain an acceptable level of rust resistance.

Isolate MB-5 has been shown to produce spores which are larger than either of the parental races. Since the spore nuclear size and number are the same as in the parents, this greater spore size would be due either to a thicker spore wall, or to more cytoplasm being present. This could confer advantage by a) making the spores more resistant to the environment, because of the thicker wall, or b) allowing a longer period of growth between germination and penetration. However, spore dispersal would be adversely affected, as larger particles are deposited before finer particles. This would result in a slower spread of infection in the field.

The generation time of isolates on a number of host varieties illustrated significant variation between isolates, which could be related to the infection-type observed, and can be taken to be indicative

of the level of host-pathogen compatibility. This was in agreement with observations in the histology section, where resistance in the host resulted in slower spread and development of the pathogen, and must affect the epidemiology of the pathogen.

The period of most rapid spread of the pathogen was between 5 and 10 days from inoculation. This corresponded closely with the time of maximum uptake of tritiated thymidine into the infected seedling leaf. When the spore nuclei were examined, the highest number of ^{3}H-labelled nuclei were observed in plants which had been labelled during this period of maximum ^{3}H uptake: the time between fleck and sporulation can therefore be correlated with intense nuclear activity and spore initiation.

Both reaction type and generation time were found to vary independently of the level of germination and penetration frequency observed.

Fusions were seen to occur between mycelial strands within the leaves, thus making plausible theories of nuclear exchange. Such fusions between different races however are only possible if two colonies are able to come into contact. Colonial size is governed by the intensity of the resistance of the host: in the OO reaction, necrosis is intense and the pathogen quickly confined, whereas in the O reaction, necrosis is less intense and not all cells are affected. This allows considerable spread of the pathogen, but is sufficient to suppress spore formation.

Contact is most likely to arise when two races to which the host is susceptible, or a race to which it is susceptible and one to which it is moderately resistant are grown together. These were the two combinations to produce recombinant isolates in this work.

If one accepts Mc Ginnis' (1956) hypothesis that chromosome number governs sexuality in Puccinia species, the observation that P. striiformis has six chromosomes provides evidence to support the assumption (Little and Manners, 1969a) that P. striiformis is heterothallic. The nuclear size, although larger than previously reported, is consistent with the number and size of the chromosomes observed.

From consideration of the above evidence, reassortment of whole nuclei would seem to be the most likely method by which race 105 E 137 arose.

The technique described to observe nuclear exchange between races could be further developed, and could provide valuable information on the nuclear behaviour of P. striiformis. The possibility of using radioactive labelled spores of one race in mixed race inoculations could prove useful, if a suitable isotope could be chosen.

It is unfortunate that the white mutant isolate was found so late in this work, as this should greatly facilitate any further work on the origins of variation in P. striiformis.

APPENDIX I

Parentage of varieties used

Cama	Cappelle Desprez x H.392.70.
Cappelle Desprez	Vilmorin 27 x Hybride du Jonquois.
Carstens V	(Squarehead Anglais x Criewener 104) x Blé de Russie.
Chinese 166	Origin China.
Compair	Chinese Spring x Aegilops comosa.
Heine 110	
Heines Kolben	Selection from Blé Saumur de Mars.
Heines Peko	Peragis x Heines Kolben.
Heines VII	Hybride à courte paille x Svalöfs Kronen.
Hybrid 46	Benoist 40 x other hybrids.
Joss Cambier	(Heines VII x Tadepi) x Cappelle Desprez.
Jubilegem	Vilmorin 23 x ?
Lee	Hope x Timstein.
Maris Beacon	(Hybrid 46 x (Cappelle Desprez x cl. 12633)) x Prof. Marchal.
Maris Envoy	(⌜(Cl. 12633 x Cappelle Desprez) x (Heine 110 x Cappelle Desprez)⌟ x Nord Desprez) x Viking.
Maris Nimrod	Sister to M. Beacon.
Michigan Amber	Identical to, or descended from, Red or Yellow Lammas.
Minister	Hybrid 40 x Prof Delos.
Moro	Pl.178383 x ?
Nord Desprez	Vilmorin 27 x Hybride du Jonquois.
P.A. 325	Transfer x Thatcher[10].
Reichersberg 42	Thatcher x Heines IV.
Riebesel 47/51	Criewener 104 x Petkuser rye (Secale cereale).

Spaldings Prolific	-
Strubes Dicckopf	Selection from English Squarehead.
Suwon 92 x Omar	-
Triticum spelta var. album	-
Vilmorin 23	(Melbor x Grosse Tête) x (Japhet x Parsel).
Thatcher	-

APPENDIX II

Culture solutions used in Chapter III.2ii.

	COMPOUND	Ml stock/litre culture
1.	$Ca(NO_3)_2.4H_2O$	0.5
2.	NH_4NO_3	0.25
3.	Buffer	0.2
4.	K_2SO_4	2.0
5.	$MgSO_4$	0.25
6.	A. FeEDTA	1.00
7.	B.	1.00
8.	C.	1.00
9.	D.	1.00

Element stock solutions.

	COMPOUND	g/2 litres
1.	$Ca(NO_3)_2.4H_2O$	472
2.	NH_4NO_3	160
3.	$K H_2PO_4$ } Buffer (pH 6)	180 g/1.5l (75%)
3.	K_2HPO_4 }	87 g/0.5l (25%)
4.	K_2SO_4	87
5.	$MgSO_4\ 7H_2O$	121
6.	(A) Fe EDTA	73
7.	(B) $Na_2B_2O_7 10H_2O$	5.2
8.	(C) $MnSO_4\ 7H_2O$	6.0
	$ZnSO_4\ 7H_2O$	2.4
	$CnSO_4\ 7H_2O$	1.8

	COMPOUND	g/2 litres
9. (D)	$Al(SO_4)_3$	0.112
	$NiSO_4\ 6H_2O$	0.112
	$Co(NO_3)\ 6H_2O$	0.112
	Li Cl	0.058
	$SnCl_2\ 2H_2O$	0.058
	KI	0.058
	KBr	0.058

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